Photosynthesis: Physiology and Metabolism

Edited by
Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer

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Advances in Photosynthesis is an ambitious book series seeking to provide a comprehensive and state-of-the-art account of photosynthesis research. Photosynthesis is the process by which higher plants, algae and certain species of bacteria transform and store solar energy in the form of energy-rich organic molecules. These compounds are in turn used as the energy source for all growth and reproduction in these organisms. As such, virtually all life on the planet ultimately depends on photosynthetic energy conversion. This series of books spans topics from physics to agronomy, from femtosecond reactions to season long production, from the photophysics of reaction centers to the physiology of whole organisms, and from X-ray crystallography of proteins to the morphology of intact plants. The intent of this series of publications is to offer beginning researchers, advanced undergraduate students, graduate students, and even research specialists a comprehensive current picture of the remarkable advances across the full scope of photosynthesis research.

The titles published in this series are listed at the end of this volume and those of forthcoming volumes on the back cover.
Photosynthesis: Physiology and Metabolism

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Preface


Oxygenic photosynthesis (covered in Volume 4 of this series, edited by Don Ort and Charles Yocum) is normally viewed as the production of reducing power by photosynthetic electron transport and the subsequent metabolism and attendant physiology made possible by this reducing power. This current volume is the first one to deal with an in-depth discussion of some of this physiology and metabolism. Taken in its broadest sense, photosynthetic metabolism encompasses almost all that happens in leaves and other photosynthetic organs. In plants with Crassulacean Acid Metabolism, even some of the metabolism that occurs at night can legitimately be considered photosynthetic. However, photosynthetic metabolism is often viewed, especially in textbooks, solely as the means by which light energy is harnessed and CO₂ is fixed, and the importance of the light-dependent metabolism of nitrogen, sulphur, lipids etc., is largely unrecognized. If omission of these aspects is regarded as a crime (particularly by those who work in these fields!), then we must plead guilty. These other areas, though vitally important, would require a volume to themselves. In this book we have concentrated on the acquisition and metabolism of carbon. However, a full understanding of reactions involved in the conversion of CO₂ to sugars requires an integrated view of metabolism. We have, therefore, commissioned international authorities to write chapters on, for example, interactions between carbon and nitrogen metabolism, on respiration in photosynthetic tissues and on the control of gene expression by metabolism. Photosynthetic carbon assimilation is also one of the most rapid metabolic processes that occur in plant cells, and therefore has to be considered in relation to transport, whether it be the initial uptake of carbon, intracellular transport between organelles, intercellular transport, as occurs in C₄ plants, or transport of photosynthates through and out of the leaf. All these aspects of transport are also covered in the book.

The principal aim of the book is to provide final year undergraduates, graduate students and researchers with an up-to-date overview of photosynthetic carbon metabolism in plants, ranging from molecular to ecophysiological aspects. The book has been divided into 24 chapters in three subsections. The first section concentrates on the pathways and the synthetic assimilation with processes such as respiration, nitrogen metabolism, transport out of the leaf, and the feedback effects of accumulation of carbohydrates. In the second section deals with the fate of that carbon, on the cellular partitioning of carbon into products, such as sucrose, sugar alcohols, starch and soluble oligosaccharides such as fructans. An overview is presented of the cellular control of carbon fixation and partitioning, including the interactions of photosynthetic CO₂ assimilation with processes such as respiration, nitrogen metabolism, transport out of the leaf, and the feedback effects of accumulation of carbohydrates. In the third section the ways in which plants acquire CO₂ from their environment are discussed. These include the difficulties of obtaining an adequate supply of CO₂ from the aqueous environment, the optimization of leaf structure, and how some plants have developed C₄ photosynthesis (to overcome the carbon loss involved in photorespiration) and Crassulacean Acid Metabolism (primarily to conserve water, but also acting as a
All these mechanisms result in advantages for these plants in certain environments.

The last time that these subjects have been comprehensively covered in a collected edition of reviews was in the early 1980s (although some aspects of the environmental regulation of photosynthesis were covered in Volume 5 of this series, edited by Neil Baker, and mentioned above). Since then there have been numerous developments, most dramatically in the molecular aspects of the subject, with knowledge proceeding apace on the structure, function and expression of the proteins involved in photosynthetic carbon acquisition and assimilation. The revolution has been such that it is now rare to find physiologists and biochemists who are not using molecular techniques or mutants and transgenic plants in their investigations. It is probably also true to say that photosynthesis and carbon assimilation have, in the intervening years, gone through a period of being unfashionable, as sinks came to be considered more important than sources in limiting crop productivity. However, nearly all plant dry mass is derived from photosynthesis and so photosynthesis and crop productivity must be linked, but the co-ordinated regulation of growth and photosynthesis makes it difficult to demonstrate control of growth by photosynthesis.

The production of this volume has involved the efforts of a large number of people, but two in particular. Firstly, the authors, who have written chapters of a high standard and who have shown considerable patience during the protracted process of assembling and editing all the contributions. Secondly, we would like to thank Larry Orr for his skill and good humor in producing the book.

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Thomas D. Sharkey
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Color Plates
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Color Plate 1. Views of the active site of spinach Rubisco showing residues involved in catalysis and binding of the metal ion (stick rendering). a, ribulose-P₂ bound to the decarbamylated (inactive), metal-free site (ER) (Taylor and Andersson, 1997); b, the carbamylated, Mg²⁺-activated site without substrate (ECM) (Taylor and Andersson, 1996); c, the carbamylated site with Ca²⁺ and ribulose-P₂ bound (ECMR) (Taylor and Andersson, 1997); d, the carbamylated, Mg²⁺-activated site with 2'-carboxyarabinitol-P₂ bound (ECM-analog) (Andersson, 1996). Heavy atoms of the phosphorylated ligands, metal ions and water molecules are rendered as balls. (See Chapter 3, p. 62, Fig. 4.)
Color Plate 2. Stereo views of the structure of spinach Rubisco. Small subunits are colored in yellow and large subunits in two shades of grey. One dark grey and one light grey subunit together comprise the basic dimeric unit of large subunits. A. Space-filling rendering of the L\textsubscript{6}S\textsubscript{8} hexadecamer showing the small subunits sandwiched between adjacent L\textsubscript{2} dimers and forming S\textsubscript{4} tetramers that cap each pole of the hexadecamer. B. Ribbon rendering of one L\textsubscript{2}S\textsubscript{2} unit showing the elements of tertiary structure with bond ligands (Mg\textsuperscript{2+} and 2'-carboxyarabinitol-P\textsubscript{2}) rendered in ball and stick and colored by atom. Reproduced from Andersson (1996) with permission. (See Chapter 3, p. 64, Fig. 6.)
Color Plate 3. Structure of the H-protein from the pea leaf glycine decarboxylase complex. Schematic ribbon representation of the overall folding of the proteins (oxidized form, methylamine-loaded form and apoprotein). The conformations of all these forms are virtually identical, consisting of seven $\beta$-strands in a sandwich structure made of two antiparallel $\beta$-sheets. Note that the lipoate cofactor attached to a specific lysine side chain is located in the loop of a hairpin configuration but following methylamine transfer it is pivoted to bind into a cleft at the surface of the H-protein. The apoprotein exhibits the same structure than the holoprotein indicating that the cofactor plays no determinant structural role. (See Chapter 5, p. 123, Fig. 3.)
Color Plate 4. Schematic diagram showing important features involved in the metabolism and transport of the primary soluble products of photoassimilation (mannitol and sucrose). The information for this diagram comes primarily from work on celery (Apium graveolens var. dulce), but the synthetic pathway has been described in other higher plant species (see text). The diagram illustrates the parallel (and presumably competitive, in terms of substrates) mannitol and sucrose biosynthetic pathways. Various documented (+/-), and possible (?) modulators of the two key regulatory steps [M6PR (2) and SPS (4)] in the mannitol and sucrose biosynthetic pathways, respectively, are listed. The diagram also illustrates the spatial separation of the anabolic and catabolic pathways for mannitol, and it is the dynamic balance between sink and source activity, translocation between them and sequestration in long and short term storage pools (involving as yet poorly described transport processes) that is ultimately responsible for regulating mannitol pools in the plant. Important enzymatic steps shown in the diagram are: 1) mannose 6P isomerase (note the roles in both anabolic and catabolic pathways); 2) mannose 6P reductase (M6PR); 3) mannitol-1-P-phosphatase; 4) sucrose-phosphate synthase (SPS); 5) sucrose-phosphate phosphatase; 6) sucrose synthase (SS); 7) mannitol-1-oxidoreductase (MTD); 8) hexokinase. Mannitol catabolism involves MTD (7), but alternative degradative enzymes exist in other species, e.g. mannitol 2-dehydrogenase (9) (see Table 1). (See Chapter 12, p. 277, Fig. 1.)
Chapter 1

Introduction

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Summary

An overview is presented of the principal mechanisms and regulatory processes involved in the acquisition of CO₂ by plants. Central to all these mechanisms is the Calvin-cycle and Rubisco, with its dual function as a carboxylase and as an oxygenase, catalyzing the first reaction in the phenomenon of photorespiration. The losses of carbon in photorespiration and the low levels of CO₂ in some habitats have led to the development of CO₂-concentrating mechanisms, such as those found in aquatic organisms and in C₄ and CAM plants. Once CO₂ is fixed into triose-P, it can then be utilized to make carbohydrates for processes such as storage and export, or used to fuel respiration and biosynthesis in the plant. The use of genetic manipulation to further our understanding of photosynthetic carbon metabolism is discussed.
I. An Overview of Pathways and Mechanisms

A. Carbon Fixation

Fifty years ago, Melvin Calvin, Andrew Benson and their colleagues embarked on the painstaking task of elucidating the pathway of CO₂ fixation in plants. The formulation of the Calvin cycle was a spectacular achievement, for which Calvin received the Nobel prize, and an account of it in a small book called *The Path of Carbon in Photosynthesis* (Bassham and Calvin, 1957) is still eminently worth reading. The Calvin cycle (also termed the Benson-Calvin cycle, the Reductive Pentose Phosphate Pathway or Photosynthetic Carbon Reduction Cycle) is the only pathway in plants which can catalyze the net fixation of carbon dioxide and it lies at the heart of carbon metabolism in leaves. (Some bacteria can reverse the Krebs cycle but this carbon reduction pathway appears to be very limited.) The discovery of the Calvin cycle was followed by the characterization of its enzymes and their regulation. One of the key discoveries of Calvin’s work was that RuBP was the primary CO₂ acceptor in plants, eventually leading to the discovery of Rubisco, which is now the most intensively studied enzyme in plant metabolism and one of the best studied proteins in plants, being one of relatively few plant proteins to have been crystallized. The remainder of Calvin cycle is increasingly studied at the molecular level, including investigations of the regulation of gene expression and its comparative biochemistry and evolution. It remains, therefore, at the forefront of photosynthesis research (Chapter 2, Martin et al.).

Rubisco has inevitably been the focus of attention in photosynthetic carbon metabolism not only because of its unique role, but also because of its inherent peculiarities and inefficiencies. Rubisco is peculiar because it contains two different subunits encoded by the nuclear and chloroplast genomes. These subunits have to be assembled within the chloroplast. The regulation and reaction mechanisms of Rubisco are also unique. These aspects are discussed in Chapter 3 (Roy and Andrews). Rubisco is an inefficient enzyme for two reasons. First, it has a low specific activity (about 3.6 μmol min⁻¹ mg⁻¹ protein). Plants therefore require large amounts of it. Rubisco can account for as much as 50% of soluble protein in the leaf of a C₃ plant and is the major investment of nitrogen in leaves. Second, Rubisco is a bifunctional enzyme in that it catalyzes both the carboxylation and the oxygenation of RuBP. Oxygenation of RuBP leads to the production of one molecule of glycerate-3-P and one of glycollate-2-P. The latter is the substrate for the photorespiratory pathway (Chapter 5, Douce and Heldt) which results in the loss of one quarter of the carbon in glycolate-2-P as CO₂. Consideration of the photorespiratory pathway provides a paradigm for the integration of photosynthetic carbon metabolism with the metabolic activities in the rest of the cell. The photorespiratory pathway involves other organelles (mitochondria and peroxisomes) besides the chloroplast, in photosynthetic carbon metabolism. Intracellular metabolite transport is, therefore, required at high rates during photosynthesis and has led to the specialized functions of organelles such as leaf mitochondria and peroxisomes. For example, glycine decarboxylase plays a dominant role in the mitochondria of leaf cells of C₃ plants, not only releasing CO₂, but also NH₃, and its sheer abundance has led to it being termed the ‘Rubisco of plant mitochondria’ by Douce. Photorespiration also has major implications for leaf nitrogen metabolism, because the rate of photorespiratory NH₃ release occurs at rates up to ten times the rate of primary NH₃ assimilation (i.e. NH₃ deriving from nitrate reduction). Since NH₃ is a far scarcer resource than CO₂, NH₃ must be very efficiently refixed by glutamine synthetase and glutamate synthase in the chloroplast.

Plants which rely solely on diffusion for carbon acquisition are called C₃ plants. In these plants, the CO₂ concentration at Rubisco is lower than that in air because of resistances to diffusion primarily at the stomata and in the mesophyll. Recent advances using stable isotope and other methods have allowed an improved estimation of the concentration of CO₂ inside chloroplasts (Chapter 14, Evans and Loreto). In C₃ plants, losses of carbon due to photorespiration can be substantial (about a quarter of the rate of CO₂ fixation at moderate temperatures) and increase at higher temperatures. Due to the relatively low affinity of Rubisco for CO₂ and the occurrence of the competing oxygenase reaction, photosynthesis in C₃ plants is not saturated with CO₂. The subtle optimization of leaf structure and stomatal function to facilitate the uptake of CO₂ by diffusion while keeping loss of water vapor to a minimum has been a major challenge to terrestrial plants and is dealt

**Abbreviations:** CAM – Crassulacean Acid Metabolism; CA1P – 2´-carboxyarabinitol-1-P; PEP–phosphoenolpyruvate; Rubisco – ribulose 1,5-bisphosphate carboxylase-oxygenase; RuBP – ribulose 1,5-bisphosphate
Some photosynthetic organisms have developed mechanisms to enhance their acquisition of CO₂ from the environment. Many aquatic photosynthetic organisms have biophysical mechanisms for the direct uptake of inorganic carbon to overcome problems caused by diffusional limitations and the pH, which govern the species of inorganic carbon (CO₂ or HCO₃⁻) available. Certain of these organisms, such as the cyanobacteria, have Rubisco with different kinetic properties and have developed a novel compartmentation of Rubisco and carbonic anhydrase in the carboxysome (Chapter 16, Badger and Spalding). Eukaryotic algae have pyrenoid bodies which may serve a similar function. This mechanism is paralleled in C₄ plants by the concentration of CO₂ in the bundle sheath where Rubisco is sequestered (Chapter 21, Sage and Pearcy).

In the case of C₄ and CAM plants, CO₂ is concentrated biochemically with resultant increases in carbon gain from the suppression of photorespiration, particularly at high temperatures, and increases in the efficiency with which resources such as nitrogen and water are utilized, which have important ecophysiological implications (Chapter 21, Sage and Pearcy). One of the principal means of studying the occurrence of C₃, C₄ and CAM plants and their water-use efficiency has been the study of the fractionation of stable carbon isotopes, which have been employed extensively both in the laboratory and in the field (Chapter 17, Brugnoli and Farquhar). Crucial to all these photosynthetic adaptations is the presence (and in some cases absence) of carbonic anhydrase (Chapter 15, Coleman), which not only plays a role in C₃ photosynthesis, but is a key component of CO₂-concentrating mechanisms in aquatic photosynthetic organisms and which can also be considered the first step of C₄ and CAM photosynthesis, since the substrate of PEP carboxylase is HCO₃⁻, not CO₂.

C₄ photosynthesis occurs mainly, though not exclusively, in the grasses. Although only about 5% of flowering plants are C₄, C₄ plants are responsible for approximately 20% of global terrestrial productivity, largely because of their dominance in savanna grasslands (Lloyd and Farquhar, 1994). Over 30 years have elapsed since the elucidation of the C₄ pathway when the ¹⁴CO₂ pulse-chase techniques employed by Calvin were elegantly pressed into service again, first by Hugo Kortschak, then by Hal Hatch and Roger Slack (for a brief discussion of the history of its discovery and some of the initial excitement engendered see Osmond, 1997 and Hatch, 1997). The C₄ pathway is an adjunct to the Benson-Calvin cycle and operates as a CO₂ concentrating mechanism that suppresses photorespiration (Chapter 18, Furbank et al.). What is particularly remarkable about C₄ photosynthesis is its integration of structure and function. C₄ photosynthesis involves the cooperation of two cell types, the mesophyll and bundle-sheath. As with photorespiration, C₄ photosynthesis requires not only intracellular transport of metabolites, between chloroplasts, cytosol, and mitochondria, but also involves rapid intercellular transport of metabolites between the mesophyll and bundle sheath (Chapter 19, Leegood). Understanding the factors that control the development of these two photosynthetic cell types and their different photosynthetic complements is a major challenge in developmental biology (Chapter 20, Dengler and Taylor), especially in view of attempts to engineer C₄-type CO₂ concentrating mechanisms into C₃ crop plants (Mann, 1999).

The C₄ pathway represents a series of variations on a biochemical theme that has almost certainly evolved independently many times. This was possible because none of the enzymes or anatomical structures involved in C₄ photosynthesis is unique to these plants. There are at least three distinct biochemical subtypes of C₄ plants, classified in accordance with the enzyme which is employed to decarboxylate C₄ acids in the bundle-sheath (NADP-malic enzyme, NAD-malic enzyme and phosphoenolpyruvate carboxykinase). However, it is becoming increasingly apparent that this is an oversimplification and that there is very considerable biochemical and structural diversity among C₄ plants. C₄ photosynthesis is thought to have evolved within the last 30 million years in response to a period of low atmospheric CO₂ concentration and high oxygen concentration following the Cretaceous period (Chapter 21, Sage and Pearcy). Ehleringer et al. (1991) have proposed that the main driving force for the evolution of C₄ photosynthesis is the increased carbon gain that results from the suppression of photorespiration. A fascinating small group of plants, C₃-C₄ intermediates, which are, to varying degrees, intermediate in structure, biochemistry and gas-exchange between C₃ and C₄ plants can be viewed as models for the evolution of the C₄ syndrome (Chapter 22, Monson and Rawsthorne).

By comparison with the C₄ pathway, the pathway
of Crassulacean Acid Metabolism (CAM) was a less dramatic discovery (for example, observations of diurnal changes in acidity in leaves of *Bryophyllum calycinum* were recorded in 1813 by Heyne (Edwards and Walker, 1983)). Although CAM is usually considered to be a CO₂-concentrating mechanism, and an extremely effective one, especially under aquatic conditions (Keeley, 1996), the prime function of nocturnal stomatal opening is the conservation of water in environments in which the water supply is unpredictable. CAM is found not only in the succulents of arid regions, but more numerous among epiphytes, such as orchids and bromeliads. While CAM is only part of a suite of physiological and structural adaptations to an erratic water supply (Chapter 24, Borland et al.), it is also clear that, as in C₄ plants, CAM has evolved many times and that the mechanisms show considerable convergence. Both C₄ and CAM photosynthesis can be considered as variations on a C₄ biochemical theme with many common regulatory mechanisms (for example, the complex regulation of PEP carboxylase, which has largely been elucidated in first CAM, then C₄ plants). Unlike C₄ plants (although see Ueno, 1998), CAM plants exhibit an extraordinary degree of photosynthetic plasticity and in many plants CAM is induced either during development or in response to environmental stress. CAM plants such as *Mesembryanthemum crystallinum* have therefore become model systems for studying the environmental regulation of gene expression and the signal transduction pathways that are involved (Chapter 23, Cushman et al.).

**B. Carbon Partitioning**

The overall reaction of the Calvin cycle can be described as the fixation of three molecules of CO₂ into a three-carbon sugar phosphate, triose-P, with the incorporation of one molecule of Pi. The principal product of photosynthetic carbon assimilation in the chloroplast is triose-P. Triose-P can be exported to the cytosol where it can be utilized to make soluble sugars, sugar alcohols (Chapter 12, Loescher and Walker), soluble oligosaccharides such as fructans (Chapter 13, Cairns et al.) or used for respiration and amino acid biosynthesis (Chapter 7, Atkin et al.; Chapter 8, Foyer et al.). Alternatively, triose-P can be retained within the chloroplast, either to make starch (Chapter 9, Trethewey and Smith) or to regenerate the initial CO₂ acceptor, ribulose-1,5-bisphosphate. Thus large-scale transport across the chloroplast envelope occurs during photosynthesis: the chloroplast is a Pi-importing, triose-P exporting organelle. These fluxes of carbon and Pi are mediated by the phosphate translocator, which catalyzes the obligatory counter-exchange of triose-P for Pi across the chloroplast envelope. The phosphate translocator is one of the best characterized of all the membrane transporters in plants (Chapter 6, Flügge). In addition, the chloroplast envelope also has transporters involved in the exchange of other metabolites such as dicarboxylates, adenylates, and hexoses, which are also involved in the turnover of carbohydrates, and the synthesis of compounds such as organic and amino acids.

There are a range of regulatory mechanisms which balance the production of triose-P by the Calvin cycle with its utilization in the synthesis of sucrose and starch or other carbohydrates. The signals involved can act either within the cell or at a distance, i.e. from sink tissues. These include the regulation of enzymes such as sucrose-phosphate synthase by phosphorylation, of the cytosolic fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate, and short-term feedback, seen as fine control by phosphate (Chapter 8, Foyer et al.). However, while there is a considerable amount of evidence that changes in Pi can act as a message between carbohydrate synthesis and triose-P production in the short-term, acting via the phosphate translocator, there is little evidence that a fine control mechanism such as this acts in the long-term, when clearly what is needed is a modulation of relative capacities of the processes of triose-P and carbohydrate synthesis. Some of this may occur by post-translational modification of enzymes, but most is likely to occur by longer-term feedback on changes in gene expression, and hence the amounts of enzymes (coarse control). It is now evident that metabolically-induced changes in gene expression, particularly those mediated by sugars, play an important role in sink-source interaction in plants (Chapter 10, Graham and Martin). This has particular relevance to changes in photosynthetic capacity that are observed after growth of plants in elevated CO₂. It is clear that, if source capacity is increased or sink capacity is restricted, carbohydrates accumulate in the source leaves, both in the mesophyll and in the vasculature (Koroleva et al., 1998). Carbohydrate accumulation may be one of the mechanisms that brings about a reduction of photosynthetic capacity. The mechanisms mediating the export of carbohydrates and amino acids from leaves are, therefore, an essential part of our
understanding of the photosynthetic process (Chapter 11, Schobert et al.), as is an understanding of the compartmentation of metabolism between and within the different cell types of leaves.

**C. Regulation and Control of Photosynthesis**

In textbooks, photosynthesis has traditionally been demarcated into ‘light’ and ‘dark’ reactions, but it has been abundantly clear from the outset (i.e. Calvin’s experiments) that photosynthetic CO₂ fixation is wholly light dependent. Indeed, it is closely integrated with electron and proton transport in chloroplasts. For example, the pH of the darkened stroma is about 7, with a free Mg²⁺ concentration of 1–3 mM, whereas in the illuminated stroma the pH is about 8 and the free Mg²⁺ concentration 3–6 mM (Leegood et al., 1985). These electron-transport-driven changes in the stroma provide an environment which is close to the optimum for the operation of the enzymes of the Benson-Calvin cycle (see, for example, Baier and Latzko, 1975) or of enzymes of C₄ photosynthesis, such as NADP-malic enzyme (Chapter 18, Furbank et al.). The enzymes of photosynthetic carbon metabolism also exhibit a wide range of allosteric responses to metabolite effectors, which are discussed in many chapters in this volume. However, there are also unique mechanisms of regulation of some of these enzymes which occur by covalent modification.

In photosynthetic cells, at least three forms of covalent modulation occur, including changes in enzyme activity brought about by the thioredoxin system and by protein phosphorylation. Rubisco activity is modulated by two unique mechanisms. In vitro, Rubisco can be activated by preincubation with CO₂ and Mg²⁺ to form an active carbamylated enzyme. This carbamylation occurs on a lysine residue (lysine²⁰⁰ of the spinach enzyme), but the CO₂ molecule involved is different from that involved in the reaction, although it plays a role in catalysis (Chapter 3, Roy and Andrews).

\[ \text{E-lys} + \text{CO}_2 + \text{Mg}^{2+} \rightarrow \text{ECO}_2 \text{Mg}^{2+} \]

Carbamylation of Rubisco in vitro occurs only in the presence of millimolar concentrations of CO₂, whereas in vivo the concentration of CO₂ in the leaf cell is only about 10 µM. Rubisco activase (Chapter 4, von Caemmerer and Quick) hydrolyzes ATP and allows carbamylation of Rubisco in the presence of physiological concentrations of CO₂ (\( K_d \text{(CO}_2) = 4 \mu \text{M} \)). Binding of RuBP to the active site prevents carbamylation and activase appears to be involved in removing bound RuBP and other ligands from the active site to allow carbamylation. Rubisco activase may also be involved in removing a tight-binding inhibitor, 2-carboxyarabinitol-1-P (CA1P; also known as hamamelonic acid 2-phosphate; \( K_d \), 32 nM) from Rubisco. CA1P is an analogue of the transition state intermediate, 3-keto-2-carboxyarabinitol bisphosphate, which occurs during catalysis (Chapter 3, Roy and Andrews). Although a CA1P phosphatase has been purified (Charlet et al., 1997), little is understood about the synthesis of CA1P and its relationship to hamamelose phosphates that are present in chloroplasts during photosynthesis (Andralojc et al., 1996), or of the role and regulation of other tight-binding inhibitors of Rubisco (Parry et al., 1997).

The activities of a number of enzymes in the chloroplast are governed by the availability of the photosynthetically-generated reductant, thioredoxin (Chapter 2, Martin et al.), providing a direct link between electron transport and carbon assimilation. Ferredoxin-thioredoxin reductase (an iron-sulfur protein with a reducible disulphide bridge) catalyzes the reduction of thioredoxin by ferredoxin. Reduced thioredoxin is able to reduce disulfide bridges on the target enzymes, modulating their activities. There are also complex changes in the regulatory properties of these enzymes because light activation can change affinities for substrates or \( V_{\text{max}} \). There are four target enzymes in the Benson-Calvin cycle: fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, ribulose-5-P kinase and glyceraldehyde-3-P dehydrogenase. Other enzymes of photosynthesis regulated by thioredoxin include NADP-malate dehydrogenase (in both C₃ and C₄ plants), the chloroplast ATPase and acetyl-CoA carboxylase. This is not a blanket means of regulation since the response to thioredoxin can be modulated by other factors, such as substrate availability. The fact that enzymes have different midpoint redox potentials (\( E_{\text{m}} \)) also means that each enzyme will have a different sensitivity to light, since a difference in \( E_{\text{m}} \) of 30 mV will represent an equilibrium constant of 10 for a two-electron transfer. Thus the ATPase could remain substantially active while the activities of the bisphosphatases change markedly (Ort and Oxborough, 1992; Hirase et al., 1999).

In the last 15 years it has become clear that phosphorylation of plant enzymes is a major influence in the regulation of carbon metabolism in the cytoplasm of leaf cells (Chapter 8, Foyer et al.).
plants. These are revolutionizing studies of the importance and role of individual proteins and of the increasing availability of mutants and transgenic enzymes for insertion into plants. A major advance in the last 10 years has been the biochemistry and by the use of knock-out mutants achieving the fine adjustment of enzyme activity to flux so that no single enzyme dominates the control of flux. However, in view of the multiplicity of regulatory mechanisms which could modulate carbon assimilation, it is also likely that they operate in a very sophisticated manner, achieving the fine adjustment of enzyme activity to diurnal variations and transients in light, CO₂, and temperature. Questions about the function of these different tiers of regulation will only be answered with a greater knowledge of the underlying biochemistry, and by the use of knock-out mutants and site-directed mutagenesis to create mutant enzymes for insertion into plants.

II. The Impact of Genetic Manipulation

A major advance in the last 10 years has been the increasing availability of mutants and transgenic plants. These are revolutionizing studies of the importance and role of individual proteins and of the control of photosynthesis and other plant processes, many examples of which are discussed in the chapters of this book. As far as photosynthetic carbon metabolism is concerned, it has led to findings which have radically changed our thinking about the operation and regulation of plant metabolism. First, it has led to the view that there is considerable flexibility in many areas of plant metabolism and this, in turn, has led to a better appreciation of the metabolic diversity of plants. In many cases, enzyme contents have been decreased by antisense technology without any immediately apparent effects on overall fluxes, growth or phenotype. Examples include the lack of any appreciable influence of the complete loss of cytosolic pyruvate kinase in leaves, though not roots (Gottlob-McHugh et al., 1992; Knowles et al., 1998), and large decreases in citrate synthase (Kruse et al., 1998) or carbonic anhydrase (Price et al., 1994) in leaves. Very often, there must be alternative pathways which allow the plant to overcome a lack of these enzymes, although it must be stressed that antisense plants do not usually lack the enzyme entirely. A good example of flexibility in photosynthetic metabolism is modulation of the activity of the phosphate translocator (Chapter 6, Flügge). A 30% reduction in translocator capacity results in no change in photosynthetic capacity, but does result in decreased export of carbon during the day, enhanced starch accumulation and carbon export at night (partly as hexose), so that, over a diurnal cycle, carbon export from the chloroplast remains undiminished. An additional important point when considering the analysis of the control exercised by enzymes over the rate of photosynthesis is that marginal, essentially unmeasurable, changes during steady state photosynthesis may, over the growth period, be compounded to result in appreciable (and measurable) differences in growth. Recent studies of tobacco plants with carbonic anhydrase reduced to 2% of wild-type levels showed that this was accompanied by no measurable changes in photosynthetic CO₂ assimilation or morphology, but it was accompanied by a decrease in the CO₂ concentration (15 mbar) at the site of carboxylation (determined from carbon isotope composition). There would be a resultant marginal (4.4%), but unmeasurable, gain in the efficiency of photosynthesis in the wild-type (Price et al., 1994). This illustrates that the assessment of control by an enzyme may be limited not only by the ability to measure resultant changes but also to employ growth conditions in which any marginal
gain becomes evident. Similar advantages may accrue from the capacity for light-regulation of enzymes, for example, in response to fluctuating light environments, which would not be evident from plant material encountering the stable light regime maintained in growth cabinets.

Second, the availability of transgenic plants has changed our concepts about the regulation and control of plant metabolism. Control analysis involves asking how much a flux changes for a given change in enzyme activity (Kacser and Burns, 1973; Fell, 1997). Highly regulated enzymes, often catalyzing non-equilibrium reactions, have been traditionally predicted to be points of control (see Stitt, 1999, for discussion). However, it has become clear that considerable reductions in the amounts of many can occur without any deleterious effects, at least when measured under growth conditions, and that the enzyme in the wild-type may exert relatively weak control because regulation can compensate for the loss of catalytic capacity. For example, only when more than 85% of the activity of ribulose-5-P kinase had been removed in transgenic tobacco plants was photosynthesis inhibited, showing that the flux control coefficient in the wild-type was zero. Amounts of ribulose-5-P, ribose-5-P, ATP and fructose-6-P rose and amounts of RuBP, glyc erate-3-P and ADP fell, all of which would tend to compensate for a decrease in total activity by activating ribulose-5-P kinase (Paul et al., 1995). In contrast, less highly regulated enzymes, such as the plastidic aldolase and transketolase, can show appreciable control because there is no means of compensating for loss of activity (Stitt 1999).

Rubisco illustrates how variable the control coefficient of a presumably important enzyme can be. A number of studies have employed transgenic tobacco plants expressing antisense against the small subunit of Rubisco to study how changing the amount of Rubisco influences photosynthetic fluxes and plant growth (Chapter 4, von Caemmerer and Quick; Stitt and Schulze, 1994). First, they show that regulation by increased carbamylation state can compensate for loss of activity, as discussed above. Second, they show that control by Rubisco depends strongly upon the environmental conditions. Third, they demonstrate complex interactions with nitrogen nutrition, such as effects on nitrate assimilation, because Rubisco represents such a large investment of nitrogen. Fourth, they show that changes in Rubisco are particularly pervasive and extend beyond carbon and nitrogen metabolism, water-use efficiency, dry matter composition, biomass allocation and whole plant growth. They demonstrate that the control by an enzyme, while it can be viewed simply within a metabolic pathway, is also part of control of processes at the level of the whole plant and that control is shared with other non-enzymic processes, such as stomatal conductance. They also emphasize that photosynthesis cannot be considered as an isolated process within the plant. However, even given the complexity of interactions, the importance of Rubisco has been confirmed by analysis of transgenic plants when Rubisco has been substantially depleted or when the light intensity is high, requiring the full complement of Rubisco.

The use of molecular studies to understand photosynthetic physiology and metabolism is now pervasive and is found in nearly every chapter in this book. This has had and will continue to have a large impact on this field.

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# Chapter 2

## The Calvin Cycle and Its Regulation

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Summary

The Calvin cycle is the starting point of carbon metabolism in higher plants. It is a typically eubacterial pathway, as comparative biochemistry of all of its enzymes from prokaryotes and eukaryotes has revealed. The structural basis of Calvin cycle function is reviewed with an attempt at a balanced consideration of biochemical and molecular findings. The structural diversity of prokaryotic enzymes is emphasized, since the genes encoding the pathway in eukaryotes have all been inherited by plants from prokaryotes through endosymbiosis. Curiously, the enzymes that constitute the pathway in different organisms are often structurally unrelated—what is conserved in evolution is merely the set of substrate conversions, not the enzymes that catalyze them. Some of the structural and regulatory properties of the enzymes were present in the antecedents of organelles, but others were newly acquired at the eukaryotic level. The expression of Calvin cycle genes is regulated by a wide spectrum of factors, though the molecular details of the regulation have yet to be unraveled. Findings that suggest the existence of multienzyme-like Calvin cycle complexes are summarized. The molecular basis of redox-modulated light regulation through the thioredoxin system and its importance for flexible control of the pathway under varying conditions is illustrated. Expression of Calvin cycle enzymes in response to external or internal stimuli is briefly reviewed, as are newer findings from the expression of antisense constructs of Calvin cycle enzymes in transgenic plants.

I. Introduction

The Calvin cycle is one of four known pathways of CO₂ fixation in nature, the three other pathways being the reverse (or reductive) citric acid cycle (Evans et al., 1966; Beh et al., 1993; Schönheit and Schäfer, 1995), the reductive acetyl-CoA (or Wood-Lungdahl) pathway (Fuchs and Stupperich, 1986; Ragsdale, 1991; Schönheit and Schäfer, 1995), and the recently discovered 3-hydroxypropionate pathway (Strauss and Fuchs, 1993; Ishii et al., 1996). However, the Calvin cycle is the only pathway of CO₂ fixation known to occur in plants (Fig. 1). It therefore figures prominently in plant biochemistry, albeit under various acronyms, among them the reductive pentose phosphate pathway (RPPP), the photosynthetic carbon reduction (PCR) cycle, the Calvin-Benson-Bassham (CBB) pathway, the Benson-Calvin cycle, the C₃ cycle, and so on. The enzymes of the Calvin cycle have been previously reviewed by Latzko and Kelly (1979), Robinson and Walker (1981) and Leegood (1990). Regulation of the Calvin cycle has been reviewed by Buchanan (1980), Macdonald and Buchanan (1990), Geiger and Servaites (1994) and, in cyanobacteria, by Tabita (1994). Historical developments surrounding the elucidation of the pathway have been briefly summarized elsewhere (Schnarrenberger and Martin, 1997). In the Calvin cycle, ATP and NADPH from the light reactions of the photosynthetic membrane are expended to reduce CO₂ to carbohydrate. From the standpoint of ATP investment per mole of CO₂ fixed, the Calvin cycle is the most costly of the four CO₂ fixation pathways known (Strauss and Fuchs, 1993). The basics of the pathway were clarified through ¹⁴C tracer studies in eukaryotic algae over 40 years ago (Calvin, 1956). The net reaction can be summarized as

\[
6 \text{CO}_2 + 18 \text{ATP} + 12 \text{NADPH} + 12 \text{H}^+ \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 12 \text{NADP}^+ + 6 \text{H}_2\text{O} + 18 \text{ADP} + 18 \text{P}. 
\]

Mutants defective in CO₂ fixation in the facultatively anaerobic, chemoautotrophic proteobacteria *Rhodobacter sphaeroides* (Gibson and Tabita, 1996) and *Ralstonia eutropha* (previously *Alcaligenes eutrophus*) (Kusian and Bowien, 1997) have been a
powerful tool for understanding the molecular biology and the genetic regulation of the pathway in these organisms. Molecular sequences are known for all of the enzymes of the pathway from spinach chloroplasts (Martin and Schnarrenberger, 1997) and from the genome sequence of the cyanobacterium *Synechocystis* PCC6803 (Kaneko et al., 1996) and, with a few exceptions, from *Rhodobacter sphaeroides* (Gibson and Tabita, 1997) and *Ralstonia eutropha* (Bommer et al., 1996). Several lines of reasoning support the view that in order to understand the Calvin cycle of higher plant chloroplasts in a broader context, it is useful to consider regulation and structural diversity within the pathway among eubacteria.

First, the pathway did not evolve de novo in plants, but rather was inherited from eubacteria via the endosymbiotic origins of organelles. As a consequence, many—but not all—of the regulatory properties that are observed among the enzymes of the higher plant pathway arose at the prokaryotic level and were simply maintained within the plant lineage, having been genetically transmitted from the cyanobacterial antecedents of plastids. The enzymes of the pathway in chloroplasts are not all acquisitions from cyanobacteria, some are acquisitions from mitochondria (Martin and Schnarrenberger, 1997; Martin and Müller, 1998) that were rerouted during evolution to a new target organelle (Martin and Herrmann, 1998).

Second, in most plastids, at least one enzyme of the pathway (one or both subunits of Rubisco) is still encoded in chloroplast DNA (cpDNA), establishing a requirement for coordination of gene expression between plastids and the nucleus in order to properly express the pathway. Indeed, the plastids of some algae even still possess the *cbbR* gene (Stoebe et al., 1998) which encodes a homologue of the transcriptional regulator of Calvin cycle gene expression in eubacteria.

Third, the quantitatively most important mechanism governing the activity of higher plant Calvin cycle enzymes—light activation via the thioredoxin
system—is present and active in cyanobacteria. This regulatory mechanism was also inherited by plants from the eubacterial antecedents of plastids, although the molecular basis for the covalent transitions in the target enzymes can differ between cyanobacteria and plants.

Fourth, although the mechanisms of Calvin cycle gene regulation in eubacteria are probably much less complex than those in eukaryotes, by no means are they irrelevant to our understanding of eukaryotic Calvin cycle gene regulation. On the contrary, due to their simplicity and tractability, mechanisms of Calvin cycle gene regulation are much better understood in eubacteria than in eukaryotes. And with the recent discovery of a cyanobacterial homologue of phytochrome (Hughes et al., 1997; Yeh et al., 1997), it appears that at least some of the basic machinery for Calvin cycle gene regulation through light in eukaryotes were simply inherited from prokaryotes through endosymbiosis, although the actual signal transduction pathways in prokaryotes and eukaryotes that lead to gene regulation through light will, in many cases, turn out to be quite different.

Finally, although the series of substrate conversions that constitute the Calvin cycle are strictly conserved across eubacteria and eukaryotes, the same degree of conservation does not apply to the enzymes that catalyze those reactions. In fact, in this chapter we will see that the pathway in proteobacteria, cyanobacteria and higher plant chloroplasts consists of enzymes that catalyze identical reactions, but, in some cases, that are altogether unrelated at the level of sequence, structure and reaction mechanism (see Martin and Schnarrenberger, 1997).

Many of the subsequent chapters in this volume deal, in one way or another, with various aspects of the Calvin cycle, including Rubisco itself (Chapters 3, (Roy and Andrews) and 4 (von Caemmerer and Quick)), metabolite transport (Chapter 6, Flügge), C4 metabolism (Chapters 18 (Furbank et al.) and 19 (Leegood)) and chloroplast-cytosol interactions (Chapters 7 (Aiken et al.) and 8 (Foyer et al.)). In this chapter, we will focus on structural, functional and regulatory aspects of the enzymes that constitute the pathway, emphasizing insights provided by molecular approaches, but considering classical biochemical aspects as well.

II. The Enzymes of the Calvin Cycle

A schematic comparison of Calvin cycle enzymes in the α-proteobacterium Ralstonia eutropha (formerly Alcaligenes eutrophus) (Bowien et al., 1993) and those encoded in the genome of the cyanobacterium Synechocystis PCC6803 (Kaneko et al., 1996a, 1996b) reveals that pathways in these bacteria comprise the same sets of substrate conversions, but in several cases with the help of enzymes that are non-homologous—or very nearly so (Fig. 2). Such structurally distinct but functionally homologous enzymes are traditionally designated as class I/class II enzymes, a term that will be used here. Differences also exist between the pathways in spinach chloroplasts and Synechocystis (e.g. use of class I vs. class II aldolase, respectively), but as depicted in Fig. 2, these differences are less grave than across the two eubacteria compared. The following sections provide a synopsis of structural and functional diversity for each Calvin cycle enzyme. Regulation of individual enzymes by covalent modification through the ferredoxin/thioredoxin system (recently reviewed by Jacquot et al., 1997b) will be considered later in this chapter.

A. Ribulose-1,5-bisphosphate Carboxylase/oxygenase

Ru1,5BP + CO₂ → 2 3PGA

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco) catalyzes the initial CO₂ fixation step. The mechanism involves an activating carbamylation reaction between CO₂ and the ε-amino group of an active site lysine residue in the large subunit (Lorimer and Mizioriko, 1980). Carbamylation is promoted by Rubisco activase (Portis, 1992). For details of Rubisco kinetics, catalytic mechanism and regulation, see Chapters 3 (Roy and Andrews) and 4 (von Caemmerer and Quick). The crystal structure of Rubisco from spinach chloroplasts is known at great resolution, it is a stout cylindrical tetramer of L₂ dimers that are ‘glued’ together by four small subunits at each end (Shibata et al., 1996; Andersson, 1996). Two structurally distinct Rubisco enzymes are known. Class I (or form I) Rubisco has a native Mr of about 560 kDa and consists of eight large subunits (LSU, Mr ~55 kDa each) and eight small subunits (SSU, Mr ~15 kDa each) comprising the L₄S₈ holoenzyme. Assembly of the heterohexadecamer requires the aid of chaperonins in both chloroplasts and eubacteria (Goloubinoff et al., 1989; Gatenby and Viitanen, 1994; Gutteridge and Gatenby, 1995).
Class II Rubisco consists only of large subunits of \( \approx 55 \) each (\( L_{2-6} \), holoenzyme) (Gibson and Tabita, 1996; Kusian and Bowien, 1997). The class I and class II large subunits share about 30% amino acid identity, indicating that they share a common ancestor. Rubisco gene diversity is a complicated matter and has been discussed in detail elsewhere (Watson and Tabita, 1996; Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic (‘green’ or G-type Rubisco) and rhodophytic plastids (‘red’ or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997).
secondary endosymbiosis (McFadden et al., 1996; Van de Peer et al., 1996) encode both subunits of R-type Rubisco in their cpDNA, and also encode a homologue of \( \text{cbbR} \), the transcriptional regulator of Calvin cycle operons in proteobacteria. The only examples in which eukaryotes have been shown to possess class II Rubisco have been described for the very diverse group of photosynthetic protists of secondary symbiotic origin known as dinoflagellates (Morse et al., 1995; Rowan et al. 1996), where, quite surprisingly, the gene for the class II Rubisco large subunit is encoded in the nucleus. The diversity of eukaryotic Rubisco genes is, in all likelihood, simply the result of sampling from ancient eubacterial gene diversity present in the common ancestor of endosymbiotic organelles, very similar to allele sampling in population genetics, but on a geological time scale (Martin and Schnarrenberger, 1997).

B. Phosphoglycerate Kinase

\[
3\text{PGA} + \text{ATP} \rightarrow 1,3\text{BPGA} + \text{ADP}
\]

Phosphoglycerate kinase (EC 2.7.2.3, PGK) catalyzes the reversible transfer of the \( \gamma \)-phosphate of ATP to the carboxyl group of 3-phosphoglycerate (3PGA), forming 1,3-bisphosphoglycerate (1,3 BPGA) for the subsequent reduction step. In all prokaryotic and eukaryotic sources studied to date, the active enzyme is a monomer with an \( M_r \) of ~44 kDa (Fothergill-Gilmore and Michels, 1993). The crystal structure of the enzyme from several sources is known. PGK is unusual in that substrate binding induces a dramatic conformational change: the two ‘wings’ of the butterfly structure are bent upon 3PGA and ATP binding by over 30 degrees, displacing distal regions of the domains by some 27 Å (Bernstein et al., 1997). The chloroplast and cytosolic isoenzymes can be separated with conventional techniques, roughly 90% of the PGK activity is localized in higher plant chloroplasts (Pacold and Anderson, 1975; Köpke-Secundo et al., 1990; McMorrow and Bradbeer, 1990). In Chlamydomonas reinhardtii, a cytosolic isoenzyme seems to be lacking (Schnarrenberger et al., 1990; Kitayama and Togasaki, 1995). Chloroplast PGK from various sources shows biphasic kinetics with \( K_{m(3\text{PGA})} \) of ~400 \( \mu \text{M} \) and \( K_{m(\text{ATP})} \) of ~500 \( \mu \text{M} \) at low substrate concentrations, with a pH optimum around 7.5 (Köpke-Secundo et al., 1990). The enzyme has not been found to be strongly regulated by allosteric effectors or by light (Leegood, 1990). The enzyme has been cloned from several higher plants (Longstaff et al., 1989; Bertsch et al. 1993) and was mapped in wheat (Chao et al., 1989). The higher plant nuclear genes for both the chloroplast and the cytosolic enzymes were obtained from cyanobacteria through endosymbiotic gene transfer (Brinkmann and Martin, 1996; Martin and Schnarrenberger, 1997).

C. Glyceraldehyde-3-phosphate Dehydrogenase

\[
1,3\text{BPGA} + \text{NADPH} + \text{H}^+ \rightarrow \text{GA3P} + \text{NADP}^+ + \text{P}_i
\]

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13, NADP*-dependent GAPDH) catalyzes the reversible reductive step of the Calvin cycle. In catalysis, 1,3BPGA forms a highly reactive thioester bond with the thiol moiety of the active site cysteine residue (Cys\(^{46}\)) under elimination of the acyl phosphate. The carbonyl group of the covalently bound intermediate is reduced to a hemithioacetal by hydride transfer from NADPH and glyceraldehyde 3-phosphate (GA3P) is released from the enzyme through cleavage of the hemithioacetal bond (Bränden and Eklund, 1980). Chloroplast NADP*-dependent GAPDH has activity with both NAD(H) and NADP(H). Although the \( K_{m(\text{NADH})} \) of the enzyme is too low to be relevant during (anabolic) CO\(_2\) fixation, NADPH being strongly preferred by the enzyme (Cerff, 1978a), recent studies indicate that the NAD*-activity may play an important role during (catabolic) ATP-synthesis in the dark (Backhausen et al., 1998). The kinetic properties are complex and depend upon the activation state of the enzyme (Cerff, 1978a; Wolosiuk and Buchanan, 1978). In the forward reaction the fully active purified enzyme has a \( K_{m(1,3\text{BPGA})} \) of roughly 30 \( \mu \text{M} \) and a \( K_{m(\text{NADPH})} \) of 40 \( \mu \text{M} \) (Baalmann et al., 1995). For the reaction in intact chloroplasts, a \( K_{m(1,3\text{BPGA})} \) of ~1 \( \mu \text{M} \) has been estimated (Fridlyand et al., 1997).

Class I and class II GAPDH enzymes are known that share only 15–20% sequence identity, both are tetramers of ~150 kDa, consisting of ~37 kDa subunits. Eukaryotes, eubacteria, and one halophilic archaeabacterium possess class I GAPDH (Pruß et al., 1994; Brinkmann and Martin, 1996). The tertiary structure of class I GAPDH is known for numerous sources (Biesecker et al., 1977; Michels et al., 1996). Class II GAPDH has been found only in archaeabacteria (Hensel et al., 1987; Fabry and Hensel,
1988; Zwickl et al., 1990). No crystal structures have been published for the class II enzyme.

Higher plant Calvin cycle GAPDH differs from all other known GAPDH enzymes in that it is an $A_2B_2$ heterotetramer rather than a homotetramer (Cerff and Chambers, 1979; Ferri et al., 1990; Scagliarini et al., 1993). The tetrameric $A_2B_2$ enzyme can reversibly aggregate to a multimeric form of 600 to 800 kDa, being less active than the dissociated form (Cerff, 1978a, 1978b; Pupillo and Faggiani, 1979; Wara-Aswapati et al., 1980; Trost et al., 1993). This multimeric form was probably the form of the enzyme first purified from plants (Yonouchot et al., 1970). Cytosolic GAPDH (EC 1.2.1.12) and non-phosphorylating GAPDH (EC 1.2.1.9), although tetramers, do not show this reversible oligomer formation (Pupillo and Faggiani, 1979). No strong allosteric effectors are known for higher plant GAPDH, but in the cyanobacterium Synechocystis PCC6803 a low MW fraction has been described that reduces the activity of Calvin cycle GAPDH in the reverse (oxidative) direction (Koksharova et al., 1998).

A novel, NAD$^+$-dependent plastid-specific GAPDH (GapCp) was recently described from Pinus chloroplasts that coexists with $A_2B_2$ GAPDH of the Calvin cycle. It shows no detectable activity with NADP$^+$ and has a $K_m$ of 62 $\mu$M and $K_m$ of 344 $\mu$M (Meyer-Gauen et al., 1994; Meyer-Gauen et al., 1998). GapCp from Pinus is possibly similar to the NAD$^+$-GAPDH reported from isolated, non-photosynthetic plastids of developing cauliflower buds (Neuhaus et al., 1993). There is also biochemical evidence for a similar NAD$^+$-specific plastid GAPDH in ripening sweet pepper fruits where, in cooperation with an NAD$^+$-dependent MDH, it appears to be important in the distribution of reducing equivalents between plastid and cytosol (Backhausen et al., 1998). In some photosynthetic tissues, for example in pine seedlings, GapCp (an NAD$^+$-specific enzyme) appears to coexist with $A_2B_2$ Calvin cycle GAPDH (NADP$^+$-specific) (Meyer-Gauen et al., 1994; Schnarrenberger, unpublished). In some non-photosynthetic tissues, GapCp may functionally replace the $A_2B_2$ enzyme. By analogy, in some photosynthetic protists, an NADP$^+$-specific GAPDH enzyme has been recruited from an NAD$^+$-specific ancestral enzyme (Liaud et al., 1997; Fagan et al., 1998). The nuclear gene for higher plant Calvin cycle GAPDH was obtained by plants from cyanobacteria, the cytosolic enzyme appears to have been obtained from the mitochondrial symbiont genome (Martin et al., 1993; Henze et al., 1995). The GapA and GapB subunits of the $A_2B_2$ enzyme arose through gene duplication during chlorophyte evolution (Meyer-Gauen et al., 1994). The B subunit is implicated in regulatory properties of the enzyme (Scagliarini et al., 1998) and possesses a CTE of roughly 30 amino acids relative to the A subunit that is involved in thioredoxin-dependent regulation (see Section VI).

**D. Triosephosphate Isomerase**

GA3P $\rightarrow$ DHAP

Triosephosphate isomerase (EC 5.3.1.1, TPI) catalyzes the rapid and reversible ketose-aldose isomerization of dihydroxyacetone phosphate (DHAP) and GA3P. The native enzyme in eubacteria and eukaryotes is a homodimer of ~27 kDa subunits (Fothergill-Gilmore and Michels, 1993), in hyperthermophilic archaeabacteria TPI is a homotetramer of 25 kDa subunits (Kohlhoff et al., 1996). The Calvin cycle enzyme of higher plant chloroplasts is a homodimer of ~27 kDa subunits (Kurzok and Feierabend, 1984; Henze et al., 1994; Schmidt et al., 1995). For both the chloroplast and cytosolic enzymes separated from leaves $K_m$ of GA3P is ~2 mM and $K_m$ is ~700 $\mu$M (Kurzok and Feierabend, 1984). The crystal structure of the enzyme from many sources is known (Velanker et al., 1997).

As for PGK, class I/class II forms of TPI have not been described. Calvin cycle TPI of higher plant chloroplasts arose through a duplication of the pre-existing eukaryotic nuclear gene for cytosolic TPI, accompanied by the acquisition of a transit peptide (Henze et al., 1994; Schmidt et al., 1995). But since the pre-existing nuclear gene was itself acquired via endosymbiotic gene transfer from ancestors of mitochondria (Keeling and Doolittle, 1997), the Calvin cycle of higher plant chloroplasts functions with TPI enzyme of mitochondrial origin that was rerouted to the plastid during evolution (Martin and Schnarrenberger, 1997).

**E. Fructose-1,6-bisphosphate/Sedoheptulose-1,7-bisphosphate Aldolase**

GA3P + DHAP $\rightarrow$ F1,6BP + H$_2$O and

E4P + DHAP $\rightarrow$ Su1,7BP + H$_2$O
Fructose-1,6-bisphosphate aldolase (EC 4.12.1.13, aldolase) catalyzes the reversible aldol condensation of dihydroxyacetone phosphate and either GA3P or erythrose-4-phosphate to yield fructose-1,6-bisphosphate or sedoheptulose-1,7-bisphosphate, respectively. Both activities are part of the Calvin cycle. Two very distinct types of aldolase enzymes occur in nature that differ in their catalytic mechanism (Rutner, 1964; Marsh and Lebherz, 1992). Class I aldolase enzymes form a Schiff-base with the substrate during catalysis via condensation of the ε-amino group of an active-center lysine residue with the carbonyl group of the substrate. Class II aldolase enzymes require divalent cations such as Zn$^{2+}$, Fe$^{2+}$ or Ca$^{2+}$ as cofactors which stabilize the carbanion intermediate formed during the reaction. The dual specificity for F1,6BP and Su1,7BP formation by aldolase applies to the chloroplast enzyme and to the cytosolic enzyme, both of the class I type in higher plants (Brooks and Criddle, 1966; Moorehead and Plaxton, 1990) and of the class II type in Cyanophora paradoxa (Flechner et al., 1999). Class I aldolases are homotetramers whereas class II aldolases are homodimers. The subunit size of both classes of aldolase enzymes is ~40 kDa, but class I and class II aldolase monomers share no detectable sequence similarity. This, in addition to the different catalytic mechanisms and unrelated crystal structures for class I (Blom and Sygusch, 1997) and class II (Cooper et al., 1996) aldolase, clearly indicates that these two classes of aldolase enzymes are the result of evolutionary functional convergence. For separated spinach chloroplast and cytosolic class I aldolase, $K_{\text{m}(\text{F1,6BP})}$ is 20 μM and 1 μM, respectively, whereas $K_{\text{m}(\text{Su1,7BP})}$ is 6 μM and 4 μM, respectively. The corresponding values for chloroplast and cytosolic class II aldolase from Cyanophora paradoxa are $K_{\text{m}(\text{F1,6BP})}$ 1 mM and 660 μM, respectively, whereas $K_{\text{m}(\text{Su1,7BP})}$ is 200 μM and 230 μM, respectively (Flechner et al., 1999).

Class I and class II aldolases have a very complex phylogenetic distribution across prokaryotes and eukaryotes (Henze et al., 1998). Most eubacteria, including all cyanobacteria studied to date, typically possess class II aldolase (Rutter, 1964; Antia, 1967), although class I aldolase is known in eubacteria (Witke and Götz, 1993). Halophilic archaeabacteria can possess either class I or class II aldolase (Dhar and Altekar, 1986). Interestingly, the Methanococcus genome does not encode a recognizable homologue of either class I or class II aldolase (Bult et al., 1996), although methanogens are known to possess aldolase activity (Yu et al., 1994; Schönheit and Schäfer, 1995), raising the possibility that a class III aldolase will eventually be found. A possible candidate for such a new class of aldolase has been described from a halophilic archaeabacterium (Krishnan and Altekar, 1991) that possesses a (mechanistically) class I aldolase consisting of 27 kDa (rather than 40 kDa) subunits with novel properties. Among higher eukaryotes, fungi typically possess class II aldolase whereas metazoa and higher plants possess class I aldolase (Schnarrenberger et al., 1990; Marsh and Lebherz, 1992; Tsutsumi et al., 1994). Euglena gracilis is exceptional among eukaryotes in that it possesses both class I aldolase (in the chloroplast) and class II aldolase (in the cytosol) (Pelzer-Reith et al., 1994b). In addition to class I and class II aldolase, ancient eubacterial gene duplications are known in class II aldolase evolution that have given rise to aldolase-related enzymes specialized for substrates other than sugar phosphates (Plaumann et al., 1997). The Calvin cycle of both proteobacteria and cyanobacteria operates with class II aldolase, while aldolase of higher plant chloroplasts is a class I enzyme, and the paucity of sequences for class I aldolase from prokaryotes makes it currently impossible to tell whence the class I gene for the chloroplast enzyme arose (Plaumann et al., 1997). To add to this conundrum of diversity, the Calvin cycle in cyanelles of Cyanophora paradoxa operates with a class II aldolase (Gross et al., 1994). Thus, Calvin cycle aldolase of plastids has arisen at least twice in evolution, and the data for class I aldolase of Euglena's chloroplasts suggest that a third independent origin of Calvin cycle aldolase in plastids is likely (Plaumann et al., 1997).

**F. Fructose-1,6-bisphosphatase**

$$\text{F1,6BP} + \text{H}_2\text{O} \rightarrow \text{F6P} + \text{P}_i$$

Fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase) catalyzes the cleavage of the phosphoester bond on C1 to yield fructose-6-bisphosphate (F6P). In most proteobacteria and cyanobacteria, the FBPase and SBPase reactions of the Calvin cycle are catalyzed by a single enzyme (F/SBPase) with dual specificity for both substrates (Gerbling et al., 1986; Gibson and Tabita, 1988; Yoo and Bowien, 1995; Paoli et al., 1995). Xanthobacter flavus is an exception (see below). F/SBPase from cyanobacteria (Gerbling et
al., 1985) is a tetramer of ~40 kDa subunits, as is FBPase from spinach (Marcus and Harrsch, 1990). The crystal structure of spinach chloroplast FBPase has been determined (Villeret et al., 1995).

FBPase catalyzes a highly exergonic reaction that is virtually irreversible under physiological conditions, and it is one of the key targets for regulation of the Calvin cycle. Activity of the enzyme is undetectable in the dark (oxidized state), but increases to maximum activities within a few minutes of illumination due to thiol reduction via the thioredoxin system (Buchanan, 1980). FBPase is specifically activated by thioredoxin \( f \) (hence the designation \( f \)) (Buchanan, 1980; Lopez-Jaramillo et al., 1997). The mechanism of chloroplast FBPase regulation was revealed by altered kinetics observed in the presence of Mg\(^{2+}\), pH, and thiols (Zimmermann et al., 1976). Activation of chloroplast FBPase by reduced thiols affects a dramatic increase of substrate affinity of >20-fold (Charles and Halliwell, 1980), \( K_{m(\text{F1,6BP})} \) of the fully activated enzyme is 6 \( \mu M \) as compared to 130 \( \mu M \) for the oxidized enzyme (Cadet and Meunier, 1988b). The sensitivity of the chloroplast enzyme to low concentrations of mercuric ions has been studied in several species (Ashton, 1998a).

Both chloroplast and cytosolic FBPase of higher plants are highly regulated (see Section VI), but by quite different mechanisms (Latzko et al., 1974; Zimmermann et al., 1976). The cytosolic enzyme is a control point for regulating flux through gluconeogenesis. Like its homologues from the cytosol of non-photosynthetic eukaryotes, it is subject to strong allosteric inhibition by AMP and regulation through F2,6BP (Stitt, 1990a, 1990b), whereas thioredoxin has no effect. The chloroplast enzyme on the other hand, is insensitive to both AMP and F2,6BP. Curiously, these distinct regulatory properties seem to have evolved specifically in the plant lineage. This is because higher chloroplast FBPase arose through gene duplication of the preexisting nuclear gene for cytosolic FBPase, that itself appears to have been acquired from mitochondria (Martin et al., 1996a; Schnarrenberger and Martin, 1997), indicating that—as in the case of TPI—the higher plant Calvin cycle functions with an FBPase enzyme of mitochondrial origin. The archaeabacteria *Methanococcus maripaludis* and *Haloarcula vallismortis* possess high FBPase activity (Altekar and Rangaswamy, 1992; Yu et al., 1994) but the enzyme has not been purified from any archaeon and the *Methanococcus* genome does not encode a recognizable gene for FBPase (Bult et al., 1996), raising the possibility that structurally unrelated class I and class II FBPase enzymes may exist.

### G. Sedoheptulose-1,7-bisphosphatase

\[
\text{Su1,7BP} + \text{H}_2\text{O} \rightarrow \text{Su7P} + \text{P}_1
\]

A highly specific sedoheptulose-1,7-bisphosphatase (EC 3.1.3.37, SPB) is not known from prokaryotes, although *Xanthobacter flavus* differentially expresses two distinct F/SBPase isoenzymes that both accept F1,6BP and Su1,7BP as substrates. The isoenzyme expressed during autotrophic growth (CbbF) has nearly equal activities with F1,6BP and Su1,7BP (3:1, respectively) as substrates, with a \( K_{m(\text{F1,6BP})} \) of 3 \( \mu M \), the other isoenzyme possesses much lower activity with Su1,7BP (van den Bergh et al., 1995). Similarly, *Synechococcus PCC 7942* possesses two immunologically distinct tetrameric FBPase isoenzymes, one of which is specific for F1,6BP, the other of which efficiently cleaves both F1,6BP and Su1,7BP (Tamoi et al., 1996). The cyanobacterial F/SBPase isoenzymes thus have slightly more similar properties to those found in higher plants, where chloroplast FBPase and SBPase in plants are separate enzymes encoded by distinct but distantly related nuclear genes (Raines et al., 1988, 1992; Martin et al., 1996a).

In contrast to FBPase and bacterial F/SBPase, which are tetramers, SBPase from higher plant chloroplasts is a dimer of ~35 kDa subunits (Nishizawa and Buchanan, 1981; Cadet et al., 1987). Also in contrast to many bacterial F/SBPase enzymes, higher plant chloroplast FBPase and SBPase show a high specificity for their respective substrates, whereby chloroplast SBPase is highly, but not completely specific for Su1,7BP (Zimmermann et al., 1976; Breazeale et al., 1978; Cadet and Meunier, 1988b). Chloroplast SBPase, as FBPase, is redox-modulated by thioredoxin \( f \) (Breazeale et al., 1978; Nishizawa and Buchanan, 1981). The reduced (activated) enzyme has a \( K_{m(\text{Su1,7BP})} \) of 50 \( \mu M \) and a \( K_{m(\text{F1,6BP})} \) of 380 \( \mu M \) (Cadet and Meunier, 1988b). But since the \( K_{m(\text{F1,6BP})} \) of reduced (activated) chloroplast FBPase is 6 \( \mu M \), the F1,6BP activity of SPB is probably of little or no physiological relevance. Similar reasoning applies to the SBPase activity of chloroplast FBPase (Ashton, 1998b). SBPase from wheat has been expressed in *E. coli* (Dunford et al., 1998), F2,6BP, a potent allosteric regulator of cytosolic FBPase, has no allosteric effect on chloroplast SBPase, but can
act as a competitive inhibitor (Cadet and Meunier, 1988b). The evolutionary relationship between chloroplast SBPase and eukaryotic FBPase and eubacterial F/SBPase enzymes is unclear (Martin et al., 1996a), but it appears that the specialization of SBPase from a bifunctional F/SBPase ancestor occurred at the prokaryotic level.

**H. Transketolase**

\[
\text{F6P} + \text{GA3P} \rightarrow \text{E4P} + \text{Xu5P} \quad \text{and} \\
\text{Su7P} + \text{GA3P} \rightarrow \text{R5P} + \text{Xu5P}
\]

Transketolase (EC 2.2.1.1, TKL) catalyzes the reversible, thiamine diphosphate-dependent transfer of a two carbon ketol group from either fructose-6-phosphate or sedoheptulose-7-phosphate (Su7P) to glyceraldehyde-3-phosphate to yield xylulose-5-phosphate (Xu5P) and either erythrose-4-phosphate or ribose-5-phosphate (R5P), respectively. TKL from various sources is a homodimer of 74 kDa subunits (Feierabend and Gringel, 1983). The crystal structure of the yeast enzyme is known (Nikkola et al., 1994; Nilsson et al., 1997). The catalytic mechanism involves nucleophilic attack of the substrate carbonyl group via the C2 carbanion of thiamine diphosphate (ThDP): the rate-limiting C2 deprotonation step requires interaction of N1' in the ThDP pyrimidine ring with Glu118 (Kern et al., 1997).

Beyond the studies of Murphy and Walker (1982), who purified the enzyme 400-fold, and Feierabend and Gringel (1983), who found only a single chloroplast species, little attention has been given to the biochemistry of this Calvin cycle enzyme. Substrate affinities for the plant enzyme have been reported as 100-130 \(\mu\text{M}\) for Xu5P, E4P and R5P (Murphy and Walker, 1982), for human erythrocytes the values 20 \(\mu\text{M}\), 30 \(\mu\text{M}\) and 2 \(\text{mM}\) were found (Himmo et al., 1989). For the purified enzyme from spinach chloroplasts 77 \(\mu\text{M}\) and 330 \(\mu\text{M}\) were found (Teige et al., 1998). TKL from the red alga *Galdieria sulphuraria* has a \(K_{\text{m}}\) of \(\sim 830 \mu\text{M}\) (J. Girnus, W. Gross and C. Schnarrenberger, unpublished). Spinach leaves appear to possess only a single RPE enzyme, localized in chloroplasts (Schnarrenberger et al., 1995). RPE has been cloned from sorghum and spinach (Nowitzki et al., 1995) and potato (Teige et al., 1995), the enzyme from spinach chloroplasts has been expressed in active form in *E. coli* (Nowitzki et al., 1995). More recently, the enzyme from spinach chloroplasts was cloned again, and was expressed in *E. coli* again (Chen et al., 1998). Neither the mechanism of catalysis nor the tertiary structure have been reported from any source. Class I / class II RPE enzymes have not been described, but three very distantly rpe-related genes exist in the *E. coli* genome, indicating the presence of relatively ancient eubacterial gene families (Nowitzki et al., 1995). The nuclear gene for higher plant Calvin cycle RPE was acquired from cyanobacteria (Martin and Schnarrenberger, 1997).

**I. Ribulose-5-phosphate 3-epimerase**

\[\text{Ru5P} \rightarrow \text{Xu5P}\]

Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1, RPE) catalyzes the reversible interconversion of ribulose-5-phosphate and xylulose-5-phosphate. RPE is a homodimer of \(\sim 23\) kDa subunits in animals (Karmali et al., 1983), *Ralstonia* (Kusian et al., 1992) and spinach (Nowitzki et al., 1995). The spinach enzyme has been cloned from sorghum and spinach (Nowitzki et al., 1995) and potato (Teige et al., 1995), the enzyme from spinach chloroplasts has been expressed in active form in *E. coli* (Nowitzki et al., 1995). More recently, the enzyme from spinach chloroplasts was cloned again, and was expressed in *E. coli* again (Chen et al., 1998). Neither the mechanism of catalysis nor the tertiary structure have been reported from any source. Class I / class II RPE enzymes have not been described, but three very distantly rpe-related genes exist in the *E. coli* genome, indicating the presence of relatively ancient eubacterial gene families (Nowitzki et al., 1995). The nuclear gene for higher plant Calvin cycle RPE was acquired from cyanobacteria (Martin and Schnarrenberger, 1997).

**J. Ribose-5-phosphate Isomerase**

\[\text{R5P} \rightarrow \text{Ru5P}\]

Ribose-5-phosphate isomerase (EC 5.3.1.6, RPI) catalyzes the reversible isomerization of ribose-5-phosphate and ribulose-5-phosphate. RPI has not been identified in the *cbb* operons of photosynthetic proteobacteria (Gibson and Tabita, 1996). No crystal
structures have been reported for this enzyme. Rutner (1970) purified RPI from spinach 2800-fold. Only a single enzyme was found, a homodimer of 23 kDa subunits, as later shown for Arabidopsis (Babadzanova and Bakaeva, 1987), that had a $K_m$(RSP) of 460 $\mu$M. Chloroplast RPI from spinach (Martin et al., 1996b) has been cloned, it has sequence similarity to RpiA from E. coli (Hove-Jensen and Maigaard, 1993). But E. coli also possesses a gene for a second functional RPI enzyme, RpiB, that is a homodimer of 16 kDa subunits. It shows no sequence similarity to RpiA, but very high similarity to galactose-6-phosphate isomerases (Sørensen and Hove-Jensen, 1996). Thus for RPI, class I (e.g. spinach RPI or $\Delta$Harrison et al., 1998). Crystal structures have been reported for class I PRK (Roberts et al., 1995; Hariharan et al., 1998). Thus for RPI, class I (e.g. spinach RPI or RpiA of E. coli) and class II (RpiB of E. coli) enzymes should be distinguished. No cytosolic isoenzyme of RPI was found in spinach leaves (Schnarrenberger et al., 1995). Calvin cycle (class I) RPI from spinach has identifiable homologues encoded in the Synechocystis and Methanococcus genomes, but due to paucity of reference sequences, the evolutionary origin of the plant nuclear gene in still unclear.

**K. Phosphoribulokinase**

$$\text{Ru5P + ATP} \rightarrow \text{Ru1,5BP + ADP}$$

Phosphoribulokinase (EC 2.7.1.19, PRK) transfers the $\gamma$-phosphate of ATP to the C1 hydroxyl group of ribulose-5-phosphate, regenerating the primary CO$_2$ acceptor. Class I and class II PRK enzymes are known (Tabita, 1994; Brandes et al., 1996a; Martin and Schnarrenberger, 1997). Class I PRK is encoded in proteobacterial $cbb$ operons. It is an octamer of ~30 kDa subunits with allosteric inhibition through AMP and allosteric activation through NADH (Runquist et al., 1995). Crystal structure data has been reported for class I PRK (Roberts et al., 1995; DHT Harrison et al., 1998). Class II PRK is found in cyanobacteria and higher plants. It is a dimer of ~44 kDa subunits in chloroplasts (Porter et al., 1986; Clasper et al., 1994). The enzyme can associate to tetramers in Synechocystis (Wadano et al., 1995). The 300-fold purified enzyme from the chromophytic protist Heterosigma carterae is a tetramer of 53 kDa subunits with a $K_m$(Mg-ATP) of 208 $\mu$M and $K_m$(Ru5P) of 226 $\mu$M (Hariharan et al., 1998). Crystal structures have not been reported for the class II enzyme. The catalytic properties of class I and class II PRK differ markedly (Tabita, 1988). The nuclear gene for the higher plant Calvin cycle enzyme was acquired from cyanobacteria (Martin and Schnarrenberger, 1997).

PRK catalyzes a highly exergonic reaction and is strongly regulated by the thioredoxin system (Buchanan, 1980). The oxidized (dark) enzyme possesses only about 2% of the activity of the fully active (reduced) form (Surek et al., 1985). Kinetic values of $K_m$(Mg-ATP) of 60 $\mu$M and $K_m$(Ru5P) of 110 $\mu$M were reported for the spinach enzyme expressed in the yeast Pichia pastoris (Brandes et al., 1996a), similar to values determined for the purified native activated wheat enzyme (Surek et al., 1985). In contrast to GAPDH, FBPase, and SBPase, thioredoxin activation of PRK does not involve lowering of $K_m$ values, but affects the $V_{\text{max}}$ (Porter et al., 1986).

**III. Calvin Cycle Gene Organization, Expression, and Regulation in Eubacteria**

Several excellent reviews on this topic have appeared recently (Tabita, 1994; Gibson and Tabita, 1996; Bommer et al., 1996; Gibson and Tabita, 1997; Kusian and Bowien, 1997; Shively et al., 1998). Mutant strains of Rhodospirillum rubrum ( Falcone and Tabita, 1993), Rhodobacter sphaeroides (Gibson et al., 1991), and Ralstonia eutropha (formerly Alcaligenes eutrophus) (Bowien et al., 1993) defective for autotrophic growth continue to uncover new genes involved in Calvin cycle function and regulation. The structure and regulation of Calvin cycle operons and gene clusters has been investigated in several eubacteria. Among eubacteria, the most complete picture of gene organization exists for the $\beta$-proteobacterium Ralstonia eutropha and the cyanobacterium Synechocystis PCC6803. The structural organization of Calvin cycle genes in these organisms could not possibly differ more.

*Ralstonia eutropha* possesses the largest $cbb$ operon characterized to date (Bowien et al., 1993; Bommer, 1996). With two exceptions (ribose-5-phosphate isomerase and triosephosphate isomerase) it encodes the entire pathway, and is transcribed as one polycistronic mRNA under the regulation of CbbR (Windhövel and Bowien, 1991), a member of the LysR family of transcriptional regulators (Tabita, 1994). The opposite extreme is realized in *Synechocystis*, where no two genes for Calvin cycle enzymes occur as neighbors in the genome (Kaneko et al., 1996a, 1996b). The *Synechocystis* genes are not separated by just a few hundred or a few thousand
bases, they are strewn around the 3.6 Mb genome with no recognizable pattern whatsoever. Even the rbcL/rbcS operon is disrupted, the genes for the two subunits being separated by an ORF of still unknown function, rbcX. *Synechocystis* possesses two genes homologous to cbbR, but neither the function of their products are known, nor whether Calvin cycle genes in *Synechocystis* form a regulon. Comparatively little is known about regulation, coordinated or otherwise, of cyanobacterial Calvin cycle genes (Beuf et al., 1994; Li and Tabita, 1994; Gibson and Tabita, 1996; Xu and Tabita, 1996).

In *Xanthobacter flavus*, cbb genes are distributed across at least two operons, the gap-pgk cluster is not contiguous with the cbb operon, but it is part of a cbb regulon under CbbR control (Meijer et al., 1996). A second cbb operon is present on a large plasmid in *Ralstonia* that is nearly identical to the chromosomal operon (Bowien et al., 1993). The cbb operons studied from *Ralstonia* (β-proteobacteria), *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Xanthobacter flavus*, and *Nitrobacter vulgaris* (all α-proteobacteria) show very little conservation of gene order across species, other than the fact that cbbR is usually transcribed, as in *Ralstonia*, on the opposite strand from a divergent promoter (Gibson and Tabita, 1996). Given the dispersed nature of the *Synechocystis* genes, it is conceivable that either these operons were assembled in independent lineages from an ancestrally dispersed state, or that fragmentation of an ancestral operon has occurred in *Synechocystis*, accompanied by rearrangements in proteobacteria. Visible rearrangement of cbb genes in α-proteobacteria suggests that considerable structural reorganization of cbb operons has occurred in these genomes during evolution. There have been reports of Calvin cycle specific activities in halophilic archaebacteria (Rawal et al., 1988; Altekar and Rajagopalan, 1990; Rajagopalan and Altekar, 1994) but the enzymes have not been characterized in detail.

A general picture of higher level control of signal transduction and gene regulation for the Calvin cycle and its integration into the general metabolism of photosynthetic eu-bacteria is beginning to emerge. The presumably top level of hierarchy involves the RegA/RegB (PrrA/PrrB) two-component sensor-kinase system (Joshi and Tabita, 1996). This system appears to integrate the control, expression and feedback of regulons for photosystem biosynthesis (Sganga and Bauer, 1992; Eraso and Caplan, 1994; Mosley et al., 1994; Allen et al, 1995), nitrogen metabolism (nif-system), and CO₂ fixation (Qian and Tabita, 1996; Joshi and Tabita, 1996). The regulatory cascade is apparently influenced by the redox state of the cells, the level of oxygen, and the presence of various carbon and nitrogen sources (Joshi and Tabita, 1996). Since precisely these factors (redox state, oxygen, carbon and nitrogen) are known to have dramatic and, in some cases, interdependent effects on plant metabolism and nuclear gene expression (Turpin and Weger, 1990; Chen et al., 1993; Escoubas et al, 1995; Kozaki and Takeba, 1996; Wingesle and Karpinski, 1996; Karpinski et al., 1997), these findings from bacterial systems may have a degree of model character for understanding related phenomena in eukaryotic systems, where the molecular basis of regulation is less thoroughly understood. Although it seems unlikely at first sight that these same prokaryotic molecular components will be found to be involved in plant signal transduction, the principles of regulatory response implemented by eukaryotic signaling/regulation machinery may ultimately prove to be very similar. A remarkable study recently provided strong evidence that some components for rapidly transducing redox signals in higher plants perceive the redox state of the plastoquinone pool directly in the thylakoid membrane (Pfannschmidt et al., 1999). Whether or not nuclear encoded bacterial two-component systems, which are still encoded in some chloroplast genomes (Stoebe et al., 1998; Martin et al., 1998), might be involved in such processes, is an attractive question.

### IV. Calvin Cycle Expression in Plants

#### A. Quantification of Activities

Regulation of the Calvin cycle enzyme expression has often been monitored using Rubisco as a marker for the pathway. PRK, TKL RPI and RPE may fulfill the same purpose, because they appear to be localized in chloroplasts exclusively as well (Schnarrenberger et al., 1995). The other enzymes may fulfill functions in other pathways. For example PGK, GAPDH, TPI, aldolase and FBPane are involved in the gluconeogenic and partially in the glycolytic reaction sequence, and the oxidative pentose phosphate pathway relies on many enzymes of the Calvin cycle (Schnarrenberger et al., 1995). Expression studies of
Calvin cycle enzymes that possess cytosolic homologues require not only measurement of total activities in crude extracts but also quantification of the amount of activities attributable to chloroplast and cytosolic isoenzymes. This is particularly necessary for the gluconeogenetic enzyme activities PGK, GAPDH, TIM, aldolase and FBPase isoenzymes, which may be separated by ion-exchange chromatography but not by gel filtration. In special cases, it is also possible to distinguish cytosol- and chloroplast-specific activities by virtue of their different substrate specificity for NADH and NADPH, as in the case of GAPDH, or by their differential response to pH, sulphydryl reagents, and Mg^{2+}, as for FBPase. But compartmentation of plant carbohydrate metabolism is not an evolutionarily conserved property across species (Schnarrenberger et al., 1990), and within a given plant it varies across developmental stages and tissues. Worse yet, across species, completely different enzymes are involved that must be assayed by different means, for example class I and class II Calvin cycle aldolase (Gross et al., 1994; Plaumann et al., 1997; Flechner et al., 1999). Thus, Rubisco is a valuable marker for regulation of Calvin cycle expression, but other enzymes may show very different regulation patterns, and sweeping generalizations to the effect that, beyond the Calvin cycle, ‘plants’ have this, that or the other pathway of sugar phosphate metabolism in this or that compartment are not possible.

The chloroplast activities of PGK, GAPDH, aldolase and FBPase in green leaves usually account for about 90% of the total activity (Heber et al., 1963; Latzko et al., 1974; Krüger and Schnarrenberger, 1983; Schnarrenberger and Krüger, 1986; Lebherz et al., 1984; Köpke-Secundo et al., 1990; McMorrow and Bradbeer, 1990), chloroplast TPI accounts for only about 50% of the total activity (Kurzok and Feierabend, 1984). For other isoenzyme activities of starch metabolism and the oxidative pentose phosphate pathway in green leaves, the cytosolic isoenzyme appears to account for most of the prevalent activity (Schnarrenberger, 1987). It appears that the activities of the regenerative part of the Calvin cycle (RPI, RPE, TKL) may not require isoenzyme separation in most cases, since they are probably located exclusively in the chloroplasts (Schnarrenberger et al., 1995), except in some specialized tissues and species (e.g. TKL in Craterostigma: Bernacchia et al., 1995). For these three enzymes no chloroplast/cytosol isoenzymes can be separated in spinach leaves (Schnarrenberger et al., 1995). It is well known that various Calvin cycle enzymes vary considerably in their maximal activities among higher plants, various algae and eu-bacteria (Smillie, 1963; Heber et al., 1967; Latzko and Gibbs, 1968; Latzko and Gibbs, 1969; Kelly and Latzko, 1979), as do activities in other pathways.

B. Expression Studies of Enzyme Activities and Transcription

The literature on expression of Calvin cycle enzymes is vast. One of the most widely studied aspects is the increase of enzyme activity and mRNA levels in response to light. The influence of light on expression of genes involved in photosynthesis has been reviewed (Chory et al., 1996; Kloppstech, 1997). Phytochromes (Schopfer, 1977; Pratt, 1995), blue light, and UV-receptors play important roles in this regulation, that ultimately reaches genes for many Calvin cycle enzymes. It is also well-known that the glycolytic, cytosolic isoenzymes of several Calvin cycle activities are, as a rule, not responsive to light and are induced under anaerobic conditions (Sachs, 1994; Kennedy et al., 1992).

Complete cDNAs have been characterized for several Calvin cycle enzymes from several sources, and all of the Calvin cycle enzymes from spinach chloroplasts have been cloned (Flechner et al., 1996; Martin et al., 1996a). Rubisco gene expression has been studied in by far the greatest detail of all of the Calvin cycle enzymes. Transcription factors involved specifically in rbcS gene expression, e.g. GT-1 (Lam and Chua, 1990; Sarokin and Chua, 1992) and GT-2 (Gilmartin et al., 1992) have been characterized. In general, expression of Calvin cycle genes in plants, particularly in etiolated seedlings, is stimulated by light. This can occur through elevated transcription, or, as recent studies of the Cen gene in Chlamydomonas mutants have shown, post-transcriptionally at the level of mRNA stability (Hahn et al., 1996). Various cis elements have been described for Calvin cycle genes from different sources, including the WF-1 element upstream of the genes for SBPase and FBPase of wheat (Miles et al., 1993), the Gap and AE boxes upstream of the Arabidopsis GapA and GapB genes (Conley et al., 1994; Kwon et al., 1994; Park et al., 1996), and an octameric motif in the first intron of the maize GapA1 gene (Donath et al., 1995; Köhler et al., 1996). The FBPase promoter also contains a DNA binding site for the GT-1 factor
which mediates light activation of expression through phytochrome in promoters of oat and rice (Lloyd et al., 1991b). In addition to the small subunit of Rubisco, for which numerous gene structures are known (Wolter et al., 1988; DeRocher et al., 1993; Fritz et al., 1993), several higher plant Calvin cycle gene structures have been characterized. These include GapA from maize (Quigley et al., 1988), Arabidopsis (Shih et al., 1992), and several other sources (Kersanach et al., 1994), SBPase and FBPase from wheat (Raines et al., 1988, 1992), aldolase from rice (Tsutsumi et al., 1994) and Chlamydomonas (Pelzer-Reith et al., 1995), and PRK from wheat (Lloyd et al., 1991a).

In studies of the transcript levels of all Calvin cycle enzymes in various tissues during spinach development (Henze, 1997), it was observed that most mRNAs were present in all green leaf tissue in roughly the same relative quantities, with the exception of rbcS mRNA, that was present at roughly 10-fold higher steady-state levels. In etiolated cotyledons, all mRNA levels were reduced at least 10- to 20-fold relative to green leaves. Upon illumination, mRNAs for rbcS, aldolase, SBPase and PRK increased within 2 h after illumination, followed by the other mRNAs. After 24 h of illumination, mRNA levels were indistinguishable from those in green leaf tissue. It is still too early to tell whether genes of the Calvin cycle in higher plants are regulated as a unit, or whether their activity is simply modulated as part of a general greening response of the gene regulatory machinery to light and redox state. In the red alga Galdieria sulphuraria, that can grow heterotrophically or autotrophically (Gross et al., 1999), isoenzymes of many Calvin cycle activities (aldolase, RPE, PGK, FBPase, and GAPDH) are specifically induced during the transition from heterotrophic to autotrophic growth (J. Girnus, C. Schnarrenberger, W. Gross, unpublished).

There are far too many reports involving expression studies of Calvin cycle genes and enzyme activities to permit thorough review. In Table 1 we have tried to provide access to some of that literature, including many studies that cannot be found by computer-searching (and omitting many studies that can). Rubisco is well known for its inducibility by phytochrome. The effects of phytochrome and other light receptors on the remaining Calvin cycle enzymes have been studied in less detail and in many of the early studies, the quantification of chloroplast vs. cytosol enzyme activities was not considered. Table 1 is by no means complete, but we hope that readers find parts of it useful.

C. Gene Regulation Through High CO₂, Sugar Sensing, and Redox State

A sugar-sensing system has been discussed in plants that may be able to significantly influence gene expression (Sheen, 1990, 1994; Koch, 1996; van Oosten and Besford, 1996; Jang and Sheen, 1997; Chapter 10, Graham and Martin). Sugars like glucose, fructose and sucrose cause strong repression of genes for photosynthetic functions, resulting in e.g. reduction of photosynthetic pigments and Calvin cycle enzymes while other sugars are largely ineffective. Glucose feeding can reduce the steady-state mRNA levels of several Calvin cycle genes in wheat, including FBPase, SBPase, PGK and rbcS (Jones et al., 1996). Among the Calvin cycle enzymes, activity and protein of Rubisco decline steadily within several days. This was demonstrated in cell suspension cultures of Chenopodium rubrum and in intact tobacco and potato leaves which were cold-girdled for 12 h to reduce assimilate export (Krapp et al., 1993), detached spinach leaves fed with glucose through the petioles (Krapp et al., 1991), mesophyll cells of tobacco (Criqui et al., 1992) and by comparing green and bleached leaves of transgenic tobacco plants expressing a yeast-derived invertase in the apoplast (Stitt et al., 1990). In some of these systems it was shown that glucose treatment resulted in the repression of these and other photosynthesis-related genes. In cell suspension cultures of Chenopodium rubrum, rbcS mRNA levels are reduced within several hours and run-on experiments with isolated nuclei indicated that also the synthesis of rbcS is reduced, as is 35S-methionine incorporation into the Rubisco protein, indicating an inhibition of de-novo synthesis (Krapp et al. 1993). In glucose-fed tobacco protoplasts and leaf discs, rbcS transcript levels are reduced within hours upon glucose addition, but rbcL transcript levels are reduced at a much slower rate (Criqui et al., 1992). Thus, regulation takes place primarily at a transcriptional level. Information on glucose repression of other Calvin cycle enzymes is limited to FBPase and GAPDH, both of which, like Rubisco, show similarly declining activities in the presence of glucose (Stitt et al., 1990; Krapp et al., 1991, 1993).

Elevated CO₂ (about 1000 ppm) can also influence
gene expression (reviewed by von Oosten and Besford, 1996), including those for some Calvin cycle enzymes. For example, whole tomato plants grown at elevated CO$_2$, relative to ambient CO$_2$-grown plants, for 10 days showed reduced Rubisco activity in the second half of this period, probably accounting for the long-term decline in photosynthetic efficiency under high CO$_2$ (Yelle et al., 1989). \textit{rbcS} transcript levels are greatly reduced in tomato plants within 4 days, while \textit{rbcL} transcript levels decline less pronounced. This effect was enhanced in detached leaves, indicating repression by elevated internal glucose levels (van Oosten et al., 1994). On the contrary, low levels of CO$_2$ resulted in an overexpression of \textit{rbcS} (Krapp et al., 1993). Besides Rubisco, also the activities of PGK and GAPDH were reduced under elevated CO$_2$ condition, though only in fully developed leaves (Besford, 1990). However, Krapp et al. (1991) observed less inhibition at high CO$_2$ than under ambient CO$_2$ at saturating irradiation and even less under low irradiation. It should be noted that any inhibition seen under these conditions might also be attributable to nitrogen limitation that can become apparent at increased growth rates (Kozaki and Takeba, 1996).

Van Oosten and Besford (1995) showed that transcript levels of plastid-encoded \textit{rbcL} and other genes involved in photosynthesis (\textit{psbA, psaAB}) were reduced in mature leaves by elevated CO$_2$. Expression of nuclear genes associated with the Calvin cycle such as Rubisco activase are also reduced by elevated CO$_2$, Expression of nuclear genes associated with the Calvin cycle such as Rubisco activase are also reduced by elevated CO$_2$ (van Oosten et al., 1994). Nuclear encoded \textit{rbcS} transcript levels were reduced in tomato plants exposed to high CO$_2$, as were plastid-encoded \textit{rbcL} transcript levels, though less markedly, and these effects could be simulated by sugar feeding (van Oosten and Besford, 1994). In terms of Rubisco content, tomato plants responded to high CO$_2$ in a manner similar to plants grown with low nitrogen supply (van Oosten et al., 1995). In bird-cherry trees grown under conditions where nutrients were not limiting, Rubisco activity decreased in response to high CO$_2$ (Wilkins et al., 1994). Clearly, there is an interdependence between CO$_2$ availability, nitrogen, redox state, sugar levels and light levels that influence gene expression. A \textit{Arabidopsis} mutant defective in a gene that might be involved in integrating or transducing sugar-related signals was recently described (van Oosten et al., 1997). Furthermore, the redox state of the thylakoid membrane itself has been recently shown to regulate the transcription of plastid genes involved in maintaining redox balance (Pfannschmidt et al., 1999), a process that certainly entails the Calvin cycle as the primary means for regenerating NADP$.^+$ The question of which and how many signaling pathways are involved in maintaining redox balance in a manner that affects the Calvin cycle is still open.

\section*{D. Regulation in Specific Systems}

A system involving preferential breakdown of 70S chloroplast ribosomes on Calvin cycle enzymes was used in early studies, because it permitted the site of enzyme synthesis to be determined long before the coding capacity of chloroplast genomes had been determined (Feierabend and Schrader-Reichhardt, 1967). Nuclear-encoded enzymes are still synthesized on 80S ribosomes and imported to chloroplasts under permissive low (22 °C) temperatures or non-permissive high (32 °C) temperatures. Among the enzymes of sugar phosphate metabolism assayed, GAPDH, PGK, TPI, TKL, FBPase, RPI, PRK and aldolase were recovered in chloroplasts at non-permissive conditions (Feierabend and Brassel, 1977; Feierabend, 1979, 1986; Feierabend and Gringel, 1983; Kurzok and Feierabend, 1983,1986; Otto and Feierabend, 1989), however, Rubisco was absent (Feierabend, 1979), transcripts of Rubisco were repressed as well (Winter and Feierabend, 1990).

Another well studied system of Calvin cycle expression is green and white leaf tissue of the ‘albostrians’ mutant of barley. This mutant shows a variegated pattern of white and green striped leaves with non-Mendelian inheritance (Hagemann and Scholz, 1962). Rubisco, GAPDH, aldolase, and FBPase were strongly reduced in white leaf tissue (Börner et al., 1976; Bradbeer and Börner, 1978; Boldt et al., 1992). In contrast, the cytosolic counterparts of the Calvin cycle enzymes, the enzymes of starch metabolism and the key enzymes of the oxidative pentose phosphate pathway were virtually unchanged (Boldt et al., 1992). Transcripts of Rubisco were totally repressed in white tissue but were enhanced in green tissue through phytochrome (Hess et al., 1991). The transcripts of chloroplast PRK, GAPDH, PGK, aldolase, and FBPase were repressed in white tissue, while those of cytosolic GAPDH and PGK were slightly enhanced (Hess et al., 1993, 1994; Boldt et al., 1994). The phenomenon is interpreted as the action of a plastid derived factor or signal which represses many (but not all) nuclear-
Table 1. Expression studies of Calvin cycle enzymes in various systems

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<th>Organism</th>
<th>Tissue</th>
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<td>Boldt et al., 1992; Hess et al., 1994</td>
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## Chapter 2 Calvin Cycle

### Table 1. (Continued)

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encoded plastid proteins in the nucleus (Boldt et al., 1990; Hess et al., 1994; Hess et al., 1997). This factor/signal also represses most genes of the glycolate pathway in plastids and peroxisomes, indicating functional unity of repression of photosynthetic functions (Boldt et al., 1997). Hahn et al. (1996) recently described a nuclear gene in *Chlamydomonas reinhardtii* that posttranscriptionally affects mRNA levels for several chloroplast proteins, including RPE.

Expression of Calvin cycle genes has been studied in the facultative CAM plant *Mesembryanthemum crystallinum* (ice plant). GAPDH mRNA accumulates in response to salt stress (Vernon and Bohnert, 1992; Vernon et al., 1993), whereby PRK expression is reduced by salt stress (Michalowski et al., 1992).
Transcript levels for cytosolic GAPDH increase during the transition from C3 to CAM metabolism (Ostrem et al., 1990). In C4 plants, only the bundle sheath cells contain a complete set of Calvin cycle enzymes (see also Chapter 2, Furman et al.).

Enzymes of the Calvin cycle can also be found in plastids of non-green tissues. An extreme example is the endosperm tissue of developing and germinating castor bean which never greens (Plaxton, 1996). Many of the plastidic isoenzymes of the Calvin cycle are present in this tissue and show an expression pattern following the typical fat-to-sugar conversion with maximum activities 5 days after germination (Nishimura and Beevers, 1981). The question is whether these enzymes really function in CO₂ fixation or whether they are due to a leaky expression. Alternatively, they could function in the oxidative pentose phosphate cycle which can provide NADPH for nitrate reduction. This has been implied by work of Emes and Fowler (1978) on TKL and transaldolase. A cytosolic class I aldolase with specificity for both F1,6BP and Su1,7BP was found in carrot storage roots but the plastidic homolog was missing (Moorhead and Plaxton, 1990). A general consideration of metabolism in chromoplasts was summarized by Smillie et al. (1995).

E. Calvin Cycle Enzymes and Expression in Euglena gracilis

Euglena gracilis can grow autotrophically and heterotrophically on various substrates (Kitaoka et al., 1989; Brandt and Wilhelm, 1990). A first screen for cytosolic and chloroplast enzyme activities of sugarphosphate metabolism was presented by Smillie (1963). Enzyme levels related to photosynthesis and degradative reactions like glycolysis seem to be antagonistically regulated under autotrophic and heterotrophic growth conditions, respectively. Most enzyme activities of the Calvin cycle increase during greening though to various degrees and decrease after transfer to heterotrophic conditions (Latzko and Gibbs, 1969; Kitaoka et al., 1989).

Enzyme activities in Euglena gracilis may also be regulated by light. This phenomenon was dissected into a blue- and a red-light reaction by the use of mutants (Schmidt and Lyman, 1974): In wild-type cells blue light increased the activities of Rubisco, PRK, chloroplast GAPDH, and chloroplast aldolase twice as effectively as red light. Mutant Y9ZNa1L had no chlorophyll and showed a blue-light but no red-light effect. Mutant Y11P22DL had small amounts of chlorophyll and showed the same activity in red and blue light. Mutant W14ZNa1L had no chloroplasts and no Rubisco but the same activity of GAPDH, PRK and aldolase as dark-grown wild-type cells, but this activity was not increased by light. In other mutants (W3BLU and W8BHL) with no plastid DNA small amounts of cytosolic class I aldolase were recorded (Karlan and Russell, 1976). Chloroplast development in Euglena gracilis is sensitive to glucose repression. When heterotrophic cells are transferred to autotrophic conditions, chloroplast development starts from proplastids. The presence of glucose inhibits greening and synthesis of Rubisco (Reinbothe et al., 1991a). During dedifferentiation from chloroplasts to proplastids, on the other hand, Rubisco synthesis ceased immediately upon transfer to the dark in the presence of glucose (Reinbothe et al., 1991b).

Chloroplast and cytosolic GAPDH are both present in Euglena gracilis cells, have been purified and show no immunochemical cross-reaction (Grisson and Kahn, 1974; Theiss-Seuberling, 1984). Chloroplast GAPDH is activated by dithiothreitol and/or thioredoxin (Theiss-Seuberling, 1981). During chloroplast development of Euglena gracilis in the light NADP-GAPDH increases in activity (Hovenkamp-Obbema and Stegwee, 1974). Both enzymes are encoded by nuclear genes and possess several unusual sequence attributes (Henze et al., 1995).

The aldolase isoenzymes of Euglena gracilis belong to the class I and class II type and are compartmented in the chloroplasts and in the cytosol, respectively (Rutter, 1964; Mo et al., 1973; Pelzer-Reith et al., 1994b; Plaumann et al., 1997). Under autotrophic conditions, the chloroplast enzyme is more active than the cytosolic enzyme (Mo et al., 1973; Karlan and Russel, 1976). This pattern is reversed during growth under heterotrophic conditions (Mo et al., 1973). While chloroplast and cytosolic aldolase of higher plants differ little in their biochemical parameters (Anderson and Pacold, 1972; Buckowiecki and Anderson, 1974; Krüger and Schnarrenberger, 1983; Leberher et al., 1984), the cytosolic class II aldolase of Euglena has a much higher $K_m$ value and a broader pH optimum than class I aldolase (Pelzer-Reith et al., 1994b). Both aldolases of Euglena gracilis show endogenous rhythmicity in the light and in the dark (Pelzer-Reith et al., 1994a; Malik, 1997). The expression of transcripts and the enzymes appears to be regulated...
posttranscriptionally (Malik, 1997). Chloroplast and cytosolic isoenzymes of TPI were separated from *Euglena gracilis* (Mo et al., 1973). The chloroplast type A enzyme is high in autotrophic and low in heterotrophic cells while the cytosolic type B isozyme predominates under heterotrophic growth conditions. An antagonistic regulation under autotrophic and heterotrophic growth conditions is also implied for the chloroplast and cytosolic FBPase when measured at pH 8.5 and 6.9, respectively (Latzko and Gibbs, 1969).

Structure and expression of nuclear genes for chloroplast proteins in *Euglena* are unusual in several respects. Many encode polyprotein precursors of chloroplast proteins (Houlné and Schantz, 1988, 1993). An example of such a polyprotein is *rbcS* in *Euglena*. The nuclear gene is transcribed as an mRNA encoding eight nearly identical concatenated small subunits that are translated as a 140 kDa cytosolic polyprotein, all eight subunits are imported into chloroplasts with the aid of a single transit peptide, and then proteolytically processed from the polyprotein into individual subunits for Rubisco assembly (Chan et al., 1990). This unusual polyprotein organization appears to be restricted to some nuclear-encoded genes in protists of secondary symbiotic origin, i.e. protists that acquired their plastids by engulfing photosynthetic eukaryotes rather than prokaryotes. [This notion was first suggested for *Euglena* (Gibbs, 1978) and subsequently demonstrated to be the case for several photosynthetic protists (Maier, 1992; McFadden et al., 1994; Melkonian, 1996; Van de Peer et al., 1996; McFadden et al., 1997)].

Such plastids are surrounded by three or more membranes instead of two, and precursor import is therefore more complex, involving ER-processing of a signal peptide prior to chloroplast uptake in the case of *Euglena* (Kishore et al., 1993; Sulli and Schwartzbach, 1996). It is likely that a number of *Euglena’s* nuclear genes for chloroplast proteins stem from the secondary symbiont and were therefore transferred twice in evolution: once from cyanobacteria to the chlorophyte nucleus, and once more from the chlorophyte nucleus to the nucleus of the *Trypanosoma*-like host (Henze et al., 1995; Plaumann et al., 1997). *Euglena’s* nuclear genes for Calvin cycle GAPDH (Henze et al., 1995), aldolase (Plaumann et al., 1997), TKL and TPI (W. Martin, unpublished) are not encoded as polyproteins, indicating that this unusual organization is restricted to certain transcripts. *Euglena’s* nuclear genes for *rbcS* and cytosolic GAPDH contain a novel class of highly structured introns that have not been described from any other eukaryotes (Henze et al., 1995; Tessier et al., 1995). Also, spliced leader sequences are found at the 5’ end of many of *Euglena’s* nuclear transcribed mRNAs (Tessier et al., 1991). Such spliced leaders have been implicated in the RNA-processing of polycistronically transcribed eukaryotic operons found in the euglenozoan lineage and in *Caenorhabditis* (Hirsch, 1994).

In other photosynthetic protists, little is known at the molecular level about Calvin cycle enzymes and gene structure, but this can be expected to change in the future since these organisms are turning up quite a number of surprising findings. For example the dinoflagellates *Gonyaulax* (Morse et al., 1995) and *Symbiodinium* (Rowan et al., 1996) use class II Rubisco in their Calvin cycle. Moreover, those class II Rubisco genes are nuclear encoded—and in *Symbiodinium* as a polyprotein, as in the case of *Euglena’s* *rbcS*. Other photosynthetic protists seem to lack chloroplast- and cytosol-specific isoenzymes of sugar phosphate metabolism. *Chlamydomonas reinhardtii* is an extreme example, since this alga has no cytosolic isoenzymes of sugar phosphate metabolism for at least eight enzyme activities, among them aldolase (Schnarrenberger et al., 1994), suggesting that the general compartmentation of carbohydrate metabolism may be surprisingly variable across protists.

V. Enzyme Interactions and Multienzyme-like Complexes

In 1970, Rutner noted that ‘...there are now several well-documented cases of multi-enzyme complexes (e.g. fatty acid synthase, pyruvic dehydrogenase [...]'), there is a tendency to implicate them in other sequential biochemical reactions’ (Rutner, 1970) and delineated some straightforward mass-activity stoichiometric difficulties encountered when such complexes are considered in the context of the Calvin cycle. Since that time, there have been many reports that some enzymes of the Calvin cycle may form multienzyme-like complexes, findings that have often been discussed in the context of metabolic channeling of intermediates. In enzymological studies prior to 1980, these complexes were rarely observed. The majority of reports deal with complexes isolated from pea and spinach chloroplasts and from green...
algeae, similar associations between Calvin cycle enzymes have not been observed in any cyanobacteria or photosynthetic proteobacteria. The reports differ substantially with respect to the number and nature of protein-protein interactions observed. There are still many open questions in this area, and there is currently no consensus concerning the nature, function or significance of such complexes. Various complexes have been isolated by ultracentrifugation in sucrose gradients, by exclusion chromatography in the presence of stabilizing compounds such as glycerol, and by ion-exchange chromatography. The multienzyme-like complexes should be distinguished from multimeric forms of individual enzymes which themselves can aggregate in purified form, for example GAPDH (Baalmann et al., 1994), FBPase (Grotjohann, 1997) or RPE (Teige et al., 1998).

Several reports concern complexes consisting of two or three enzymes. A complex containing Rubisco, RPI (90 kDa) and PRK (54 kDa) was reported from pea leaves (Sainsis and Harris, 1986) that catalyzed R5P-dependent CO2 fixation in the presence of ATP, and contained about 4–5% of RPI and PRK activities in the complexed form. In a similar complex from spinach, 75% of PRK and 7% of RPI were found to associate and copurify with Rubisco (Sainsis et al., 1989). The ratio of PRK to Rubisco was estimated to be 1:1 to 1:3. In another report, a complex of PRK with GAPDH was isolated from Scenedesmus obliquus (Nicholson et al., 1987). The stoichiometry was estimated to be GAPDH4,PRK4 with a (too low) molecular mass of 560 kDa. Also, PRK and GAPDH were found to form a complex coexisting with the free enzyme forms in spinach (Clasper et al., 1991). A novel, 12 kDa chloroplast protein (CP12) has recently been described from higher plants that shares high sequence similarity with the CTE of the GapB subunit (Pohlmeyer et al., 1996). CP12 interacts with GAPDH in affinity chromatography and with PRK in the yeast two-hybrid system. Under oxidizing conditions, CP12 interacts with both proteins to form ~600 kDa complexes and has been suggested to be involved in regulation (Wedel et al., 1997; Wedel and Soll, 1998).

A complex was isolated from Chlamydomonas reinhardtii consisting of PRK4,GAPDH4 with an average molecular mass of 460 kDa (Avilan et al., 1997; Lebreton et al., 1997), equal to the sum of individual masses of the free enzymes. The dissociation of the complex can be achieved by reducing agents like DTT, NAD(P)H, reduced ferredoxin or reduced thioredoxin, accompanied by an increase particularly in PRK activity. PRK is inactive in the oxidized form (see Section VI) and gained some activity during complex formation with GAPDH in a manner similar but not identical to chaperonin action (Lebreton et al., 1997). However, this increase corresponds to only a few percent of the activity of the reduced form present in the light. The subsequent dissociation of the complex by reducing agents causes a conformation change in PRK, another 20-fold increase in PRK activity with a 4-fold decrease in Km(R5P) and a 2-fold decrease in Km(ATP). During complex dissociation, GAPDH showed a relative increase in favor of NADP+ over NAD+ activity (Avilan et al., 1997). On the other hand, the complex could form spontaneously, upon addition of NAD+ or oxidized glutathione (Avilan et al., 1997; Lebreton et al., 1997). The association between GAPDH and PRK in Chlamydomonas involves two enzymes that catalyze non-consecutive steps in the pathway and the complex is present under dark conditions where there should be no Calvin cycle activity. Thus, the complex is unlikely to be involved in channeling in the classical sense (Gontero et al., 1994; Ricard et al., 1994).

There have been reports of larger Calvin cycle multienzyme-like complexes involving several additional enzymes. The first such larger complex contained PRK, Rubisco, PGK, and GAPDH and was reported by Müller (1972), who recognized that the fragile complex is dissociated by NADPH or ATP and that the enzymes involved are activated during dissociation. The complex had a molecular mass of 400 kDa, less than the value of 700 to 800 kDa expected. Sasajima and Yoneda (1974) found that RPI, TKL and RPE copurify. More recent reports have detected complexes with an M, in the range of 500 to 1000 kDa (Gontero et al., 1988; Gontero et al., 1993; Rault et al., 1993; Sainsis and Srinivasan, 1993; Süss et al., 1993, 1995). The enzymes involved in these complexes and their stoichiometry differ in individual laboratories, the function is generally interpreted as metabolic channeling.

Gontero et al. (1988) found a complex consisting of Rubisco, RPI, PRK, PGK and GAPDH. The complex was fairly stable and homogeneous during ultracentrifugation. DTT increased the activity of the individual enzymes. The complex catalyzed CO2 fixation with R5P, ATP, NADPH and CO2. The molecular mass of the complex was estimated as 520 kDa and the ratio of the individual enzymes inferred
to be 2PRK:2GapA:2GapB:2RbcS:4RbcL, in addition to some RPI and PGK (Rault et al., 1993). Because the molecular mass of the individual enzymes is anticipated to be much larger than that of the complex, it was suggested that Rubisco might exist in an $L_nS_m$ form (Rault et al., 1993), differing from that of the crystallized enzyme (Shibita et al., 1996). If the complex is subjected to SDS-PAGE, several protein bands are observed corresponding to the bands of the individual enzymes.

A complex isolated from spinach contained Rubisco, PRK, GAPDH, SBPase, ferredoxin-NADP reductase (FNR) and chaperonin 60 (Süss et al., 1993) and fixed CO$_2$ from R5P. The complex was stable at low salt conditions (<200 mM KCl) and dissociated under high salt conditions (>250 mM KCl). Ammonium sulfate (1 M) or pH 4.5 completely dissociated the complex. All enzymes of the complex were found almost exclusively attached to the outer surface of thylakoid membranes during gold immunolabeling except Rubisco, which showed also stromal localization (Süss et al., 1993a, 1993b; Adler et al., 1993). Similar association with thylakoids had previously been reported for Rubisco (Grisson and Kahn, 1974; McNeil and Walker, 1981), PRK (Fischer and Latzko, 1979) and GAPDH (Grisson and Kahn, 1974). A newly described ~600 kDa complex from tobacco contained Rubisco, PRK, RPI and carbonic anhydrase (Jebanathirajah and Coleman, 1998).

In other reports, complexes consisting of PGK-GAPDH (Malhotra et al., 1987; Macioszek and Anderson, 1987; Macioszek et al., 1990), GAPDH-TPI, aldolase-TPI, GAPDH-aldolase (Anderson et al., 1995), and PRI and PRK (Anderson, 1987; Skrukrud et al., 1991) have been found. Cytosolic PGK and GAPDH were also found to form a bienzyme complex (Weber and Berhard, 1982; Malhorta et al., 1987). Complexes of aldolase-GAPDH, PGK-GAPDH and aldolase-TPI from chloroplasts were isolated and characterized in pea (Anderson et al., 1995). It has been suggested that interaction among GAPDH, TKL and aldolase around SBPase may lead to a direct transfer of GA3P among these enzymes (Marques et al., 1987). Finally, PRI and PRK were shown to have kinetics in a complex state that differed from those anticipated for substrates used by non-complexed enzymes (Anderson, 1987). The theoretical kinetics of Calvin cycle multienzyme complexes have been modeled (Gontero et al., 1994; Ricard et al., 1994). In multienzyme complexes, the kinetics become increasingly complicated because of the combined action of several enzymes.

Note that the sum of molecular weights of the native enzymes from spinach chloroplasts shown in Fig. 1 is about 1500 kDa, Rubisco alone contributing a third of that. Since Rubisco is far more abundant than any of the other Calvin cycle enzymes in plastids, it is clear that not all active Calvin cycle enzymes can exist in a complexed state (Rutner, 1970).

Summing up these findings on Calvin cycle multienzyme complexes, it appears clear that interactions between various enzymes do exist, but there is no consensus on which or how many enzymes interact and whether the same enzymes interact in different species. The metabolic relevance of these complexes is still unclear. Pressing problems concerning these associations have yet to be solved.

First, the isolated enzyme complexes are usually described to *dissociate* in the presence of reducing thiols (light), but the key regulatory factor of overall Calvin cycle activity is light-mediated (redox) *activation* through reduced thiols (see below). This discrepancy is difficult to reconcile with metabolic channeling, since the kinetic data indicate higher enzyme activities for associated enzymes via transfer of substrates in a consecutive reaction sequence, but flux through the pathway in the dark is basically nil (associated state) due to severe down-regulation of FBPase, PRK and SBPase (and moderate down-regulation of GAPDH) in the dark (i.e. in absence of reduced thioredoxins). But if the overall activities of the complexed enzymes are higher, as many such studies indicate, we are left with the question of the physiological relevance of improved kinetics for (dark-) associated enzyme complexes, since the active forms are dissociated in the light (see Section VI).

Second, if associations between enzymes are as critical to Calvin cycle function as the interpretations of many multienzyme studies would suggest, then problems in understanding the pathway ensue when the results from antisense inhibition of Calvin cycle enzymes are considered (see section VII). This is because antisense studies have shown that most (but not all) Calvin cycle activities must be reduced on the order of five- to ten-fold to affect a significant reduction in assimilation rate under normal growth conditions. If the brunt of assimilation occurs in complexes, limiting any one component should be expected to have a more drastic effect. Further work is needed to clarify the general significance of these enzyme association phenomena.
VI. Biochemical Regulation in Chloroplasts

Light governs not only Calvin cycle gene expression in higher plants, it is also the foremost determinant of enzyme activity, and hence flux through the pathway (Buchanan, 1980; Wolosiuk et al., 1993). Light regulation of the Calvin cycle is achieved by modulation of enzyme activity of four enzymes through the ferredoxin/thioredoxin system: FBPase, SBPase, PRK and GAPDH (Buchanan, 1991). The first three enzymes are obvious targets for regulation, since they catalyze reactions that are irreversible under physiological conditions, their regulatory principle is the same: reduced activity as a result of oxidation of regulatory cysteines by $O_2$ in the dark, full activity through reduction of regulatory cysteines by reduced thioredoxin in the light. Activity is continuously adjusted in the light through continued reoxidation ($O_2$) and reduction (thioredoxin). The steady-state between these interconvertible enzyme forms is individually influenced by metabolites (Scheibe, 1990). The fourth reaction, catalyzed by GAPDH, is reversible. Its activity is dependent upon its state of aggregation, redox modulation being the prerequisite enabling this metabolite-induced interconversion under physiological conditions.

A. The Ferredoxin/Thioredoxin System

Ferredoxin (Fd) reduced by photosynthetic electron flow provides electrons for $\text{NADP}^+$ reduction via Fd/ NADP reductase, for nitrite reduction via nitrite reductase, for sulfite reduction via sulfite reductase, for the reductive generation of glutamate from oxoglutarate via glutamate synthase (GOGAT), and for the reduction of the thioredoxin (Td) via ferredoxin-thioredoxin reductase (FTR) (reviewed by Buchanan, 1980; Woodrow and Berry, 1988; Scheibe, 1990; Buchanan, 1991; Knaff and Hirasawa, 1991; Wolosiuk et al., 1993; Jacquot et al., 1997b). FTR is composed of two different subunits, subunit A is rather variable between organisms, subunit B is more highly conserved and contains an Fe-S cluster in addition to conserved cysteines involved in redox transfer, but it is not a flavoprotein (Tsugita et al., 1991; Falkenstein et al., 1994). Thioredoxins are small heat-stable proteins that occur in all organisms and in many compartments. In the chloroplast various isoforms occur that differ in their primary structures and specificities: Tdm, Tdf, and Tdf (reviewed by Eklund et al., 1991). Invitro, Tdm primarily activates NADP-dependent malate dehydrogenase (NADP-MDH), and inactivates chloroplast glucose-6-phosphate dehydrogenase (G6PDH), while Tdf preferentially activates chloroplast FBPase, SBPase, PRK, GAPDH in addition to the chloroplast coupling factor CF1 (reviewed by Buchanan, 1991). Whether this pattern of specificities also holds in stroma, where the protein concentration is very high, remains to be established.

Traditionally, light/dark modulation of chloroplast enzymes was considered as an all-or-nothing ‘on/off’-switch, but more recently it has become apparent that it is also a means to fine-tune enzyme activities in the light (Scheibe, 1990, 1991,1995). This is because $O_2$ present at high concentrations in the chloroplast (Steiger et al., 1977) continuously reoxidizes the cysteines generated by thioredoxin-mediated electron flow to the target enzymes. Light-modulated enzymes thus exist as two interconvertible enzyme forms that are subject to covalent modification (reduction and reoxidation of cystine/cysteine residues), comparable to those enzymes that are subject to protein phosphorylation/dephosphorylation (Scheibe, 1990). In both cases, energy is consumed to drive the cycle between the two forms, but in the light, energy in the form of reducing equivalents is abundant and poses no significant drain on the photosynthetic membrane.

Target enzymes as well as the chloroplast thioredoxins are characterized by the very negative midpoint redox potentials of their regulatory cysteines (Faske et al., 1995). For NADP-MDH, FBPase and PRK these are around −380 mV, similar to that of the nonphysiological reagent dithiothreitol (DTT), and even more negative than the value of −350 mV for Tdm and Tdf (Gilbert, 1984). These redox potentials are all more negative than those of NADP(H) (−320 mV), and of glutathione (−260 mV), indicating that these protein thiols cannot be reduced by cellular reductants other than reduced ferredoxin. In some cases, mixed disulfides can be formed with low molecular weight thiols such as glutathione (Ocheretina and Scheibe, 1994). That certain chloroplast proteins occur in an oxidized form is a rather special attribute, since usually only extracellular proteins tend to exhibit this property (Fahey et al., 1977). For chloroplast enzymes it is this specific property which is the basis for a very flexible regulatory system.
B. Target Enzymes

The light/dark-modulated chloroplast enzymes are characterized by their unusually negative redox potentials. As a result of this, they are only in the reduced state when electrons of very negative redox potential from ferredoxin are available in the light; otherwise they relax to their oxidized state. The redox potentials themselves, however, are subject to change by specific metabolites, mostly the substrate or the product of the respective enzyme reaction. These metabolites are also known to act as effectors of the reductive and/or the oxidative part of the redox cycle. At equilibrium (with the redox buffer, in vitro) or at steady-state (in vivo) changes in the relative effector concentrations result in a more or less pronounced shift of the ratio between oxidized and reduced enzyme form (in a concentration-dependent manner) (Faske et al., 1995). Redox-modulated chloroplast enzymes generally exhibit high similarities with their non-redox-modulated homologues from other sources, but also tend to possess unique, cysteine-bearing sequence motifs that are responsible for their regulatory properties (Scheibe, 1990).

Chloroplast FBPase is the classical target for light regulation via thioredoxin (Buchanan, 1980). There is a strong dependence of FBPase activity upon the F1,6BP concentration, i.e. FBPase cannot easily be activated by DTT (or in the light) in the absence of F1,6BP. The resulting regulatory pattern is a strict feedforward mechanism of FBPase activation due to increasing F1,6BP levels (Scheibe, 1991). Several studies have investigated the mechanism of activation using FBPase overexpressed in E. coli (Jacquot et al., 1995; Hermoso et al., 1996; Jacquot et al., 1997a; Lopez-Jaramillo et al., 1997; Sahrawy et al., 1997). Chloroplast FBPase possesses a conspicuous insertion of 12–15 amino acids in the central region of the primary structure with two conserved cysteine residues separated by five amino acids (C173 and C178) preceded by a third conserved cysteine (C155) further N-terminal (Marcus et al., 1988; Raines et al., 1988). In vitro mutagenesis of C173 and C178 results in enzymatically active FBPase enzymes that can no longer be regulated by thioredoxin, indicating that these may be specific targets of thioredoxin regulation (Jacquot et al., 1995). But in a more recent study, C155 was also found to be responsible for redox dependence (Jacquot et al., 1997a). Replacing these three cysteines with serine residues in rapeseed FBPase also resulted in enzymes that were active in a manner largely independent of redox modulation (Rodriguez-Suarez et al., 1997). It is not yet clear how redox modification of these three target cysteines modulates FBPase activity at the mechanistic level. For spinach FBPase, it appears that appears that only a single disulfide bridge is formed, that between C155 and C174, as shown by mutant protein studies (A. Reichert and R. Scheibe, unpublished). Binding of thioredoxin to FBPase may be mediated by electrostatic interactions (Mora et al., 1998).

Pea Tdm overexpressed in E. coli can activate spinach FBPase, in contrast to previous findings, and K<sub>m</sub>Emutagenesis of pea Tdm leads to a 50% decrease in FBPase activation (Lopez Jaramillo et al., 1997). A poorly conserved region of the FBPase alignment is found immediately preceding the redox regulatory cysteines. Deletion of this region yields active FBPase that can no longer be redox-regulated (Sahrawy et al., 1997). The same deleted region was expressed in E. coli as a 19 amino acid fragment (P<sub>A09</sub>–G<sub>G67</sub>), and shown to strongly interact with pea Tdm and, surprisingly, to increase the efficiency of Tdm in FBPase activation (Hermoso et al., 1996). These findings suggest that this may be the Td docking site that causes a conformational change upon binding.

Chloroplast GAPDH is among the first enzymes for which light regulation was known (Marcus 1960; Ziegler and Ziegler, 1965) and where a posttranslational modification was thought to modulate activity changes (Müller et al., 1969). Light/dark modulation of the enzyme correlates with changes in its aggregation state in isolated intact spinach chloroplasts. In the dark, only the less active, ~600 kDa form of chloroplast GAPDH appears to be present. In the light, the 150 kDa (tetrameric) form with a specific activity on the order of 120 U·mg<sup>−1</sup> (Tröst et al., 1993; Scagliarini et al., 1993; Baalmann et al., 1994, 1995) predominates. Conversion to the activated 150 kDa form under reducing conditions (reduced thioredoxin) is achieved in the presence of low 1,3-bisphosphoglycerate (1,3BPGA) concentrations (K<sub>s</sub> = 1-2 μM) and is accompanied by a 20-fold increase of the affinity for the substrate 1,3BPGA (Baalmann et al., 1994, 1995). In the presence of reduced thioredoxin, this transition is modulated by low concentrations of 1,3BPGA and is accompanied by a 20-fold increase of the affinity for 1,3BPGA as a substrate. The oxidized form can only be activated at unphysiologically high concentrations of the effector 1,3BPGA (Baalmann et al., 1994; 1995). Aggregation of active chloroplast GAPDH to ~600
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kDa forms can also be induced in vitro by addition of 140 μM NAD⁺ (Pupillo and Giuliani-Piccari, 1975; Cerff, 1982). The mature A and B subunits of spinach chloroplast GAPDH expressed in E. coli associate to active A₄ and B₄ forms, as does the derivative of the B subunit lacking the CTE, which is four-fold more active than the B₄ form (Baalmann et al., 1996). These findings indicate that aggregation-mediated activation is dependent upon the 30 amino acid long carboxy-terminal extension (CTE) of the B subunit, consistent with independent findings from partial proteolysis studies (Scheibe et al., 1995). The two conserved cysteines in the CTE may be the targets for thioredoxin reduction, but other models have been proposed (Pacold et al., 1995).

PRK is activated by incubation with reduced thioredoxin and inactivated by oxidation. The target cysteine residues involved in Td regulation of spinach chloroplast PRK were identified through chemical modification as C₁₆ and C₅₉, the latter being close to the ATP-binding site (Porter et al., 1988), findings which were substantiated through in vitro mutagenesis and expression of the active enzyme in the yeast Pichia pastoris (Brandes et al., 1996a, 1996b). Although the cosubstrate and effector ATP decreases the rates of both activation and inactivation, it does not significantly influence the ratio between oxidized and reduced PRK at equilibrium (Faske et al., 1995). Therefore, redox-activation of PRK in the light occurs in a metabolite-independent manner, whereby the activated enzyme is then subject to non-covalent regulation of its catalytic activity by various metabolites (Gardemann et al., 1983).

Chloroplast SBPase, like FBPase, is enzymatically inactive in the oxidized state, but activity is restored within minutes by reduced sulfhydryls, thioredoxins f₈, f₉ and m can activate the enzyme (Cadet and Muenier, 1988a). Although SBPase and FBPase are related enzymes, the central insertion containing the redox-responsive cysteine residues of FBPase is, surprisingly, lacking in SBPase (Raines et al., 1992). Results from molecular modeling have suggested that interdomain disulfides might be involved in chloroplast SBPase redox regulation (Anderson et al., 1996). The target cysteines for SBPase regulation via thioredoxin have been identified through mutagenesis studies (Raines et al., 1998). It has also been suggested that flux through SBPase might be limiting for flux through the pathway (Pettersson and Ryde-Pettersson, 1989).

Oxidation of cysteines is a well-known reaction occurring upon folding and secretion of proteins, often assisted by the action of thioredoxin-like proteins such as protein-disulfide isomerase (for review see: Loferer and Hennecke, 1994). However, these reactions are essentially irreversible and confer stability to the proteins in an oxidizing extracellular environment. The fact that intracellular proteins are oxidized in a reversible manner is unique to photosynthetic systems. Oxidation is accompanied by drastic changes of particular enzymic parameters as Vₘₐₓ, Kₘ and Kₘₐ₃, thus fulfilling important criteria of a covalent modification likely to be of relevance in vivo (Ziegler, 1985). For PRK, oxidation results in a decrease of its Vₘₐₓ to essentially zero. For FBPase and SBPase, oxidation decreases affinity for sugar bisphosphate and Mg²⁺ and shifts the pH optimum to higher values. Oxidation of Calvin cycle GAPDH results in the requirement for increased activator (1,3BPGA) concentrations (Trost et al., 1993; Baalmann et al., 1994), whereby 1,3BPGA is responsible for the dissociation of the aggregated low-affinity form to generate the high-affinity A₂B₂ heterotetramer.

The electron pressure required for the reduction of GAPDH and of PRK is rather low when compared to FBPase and SBPase, so that electron flow through thioredoxin will not be limiting even at low light, thus enabling the reductive step of the Calvin cycle and the regeneration of the CO₂ acceptor to occur under all conditions. Flux through these steps is thus determined by the availability of substrates. The chloroplast isozymes of glucose-6-phosphate dehydrogenase (G6PDH), ATPase and NADP-MDH are also subject to covalent redox-modification, but will not be considered here.

C. Physiological Consequences

Actual enzyme activities in isolated chloroplasts achieved at various light intensities will strongly depend on the metabolic status. Chloroplast GAPDH activity is at 100% even at low light intensities when ATP and 3PGA are present, since they readily generate 1,3BPGA concentrations required for GAPDH activation through the (unregulated) PGK reaction (Baalmann et al., 1994). The actual activity, however, will be lower due to the lack of 1,3BPGA as a substrate (Friddlyand et al., 1997). Thus the flux at this step is always adjusted to the overall flux through the Cycle. Flux through the PRK reaction in the light is also determined by substrate levels, since the enzyme
is in the fully active form at all light intensities (Scheibe, 1995). In contrast, the activation state of FBPase is strongly dependent upon the presence of F1,6BP which promotes reductive activation and inhibits oxidative inactivation (Scheibe, 1995). In intact chloroplasts, any decrease of electron pressure induced by the addition of electron acceptors decreases FBPase activation and leads to increased F1,6BP levels that apparently are not sufficient to support activation under these conditions (Holtgrefe et al., 1997), indicating that a strict control of CO₂ fixation is exerted by redox state and energy charge.

Correlations of light intensity and photosynthetic electron transport on the one hand, and stromal enzyme activities on the other, have been analyzed (Harbinson et al., 1990; Sassenrath-Cole et al., 1994). In some cases, a more or less positive correlation was found. But in others, as at low O₂ or CO₂ concentration, this did not appear to be the case (Harbinson and Foyer, 1991; Sassenrath-Cole et al., 1994). Under some conditions, the metabolite levels will also be altered, in turn shifting the activation states of the enzymes at a given thylakoid redox state. Therefore, it is important to include both electron pressure, metabolite levels and enzyme concentrations in vivo (Harris and Koniger, 1997) into models of photosynthetic regulation, since the redox potential of the isolated enzyme alone does not reflect the dynamic situation in vivo (Kramer et al., 1990).

Taken together, the differential light- and metabolite-dependent modulation of redox-modulated enzymes provides the basis for an extremely flexible fine-tuning. In isolated chloroplasts, this mechanism has been shown to establish a strict hierarchy between all reactions consuming light-generated electrons, so that no competition between the various pathways will occur (Backhausen et al., 1994). Due to the differential affinity of the various electron acceptors for electrons from reduced ferredoxin, the essential reactions as NADP⁺ reduction, nitrite reduction and thioredoxin-dependent enzyme activation will preferentially occur under physiological conditions. The required ATP/NADPH ratio is adjusted by the action of the malate valve that is controlled by the light/dark-modulated NADP⁺-malate dehydrogenase present in all chloroplasts studied to date (Backhausen et al., 1994). Only an excess of electrons will flow into the Mehler/ascorbate reaction and into cyclic electron flow (Steiger and Beck, 1981; Heber and Walker, 1992).

In contrast, at higher concentrations the unphysiological electron acceptors H₂O₂ and nitrite can drain electrons from the Calvin cycle (Robinson et al., 1980; Backhausen et al., 1994) and thus inhibit photosynthesis.

### VII. Studies of Calvin Cycle Enzymes with Antisense RNA

The effects of antisense RNA on carbohydrate partitioning in plants (Sonnewald et al., 1994; Frommer and Sonnewald, 1995; Furbank and Taylor, 1995; Stitt and Sonnewald, 1995) and on general aspects of regulation (Furbank and Taylor, 1995) can also be found throughout this volume. Antisense RNA rarely results in a complete inhibition of gene expression, residual gene expression can often supply sufficient enzyme activity to maintain a wild-type phenotype (Furbank and Taylor, 1995), and altered phenotypes of plants with reduced levels of Calvin cycle enzyme are sometimes only visible under conditions where factors such as light, CO₂ or mineral nutrition are limiting. Antisense technology has opened up new avenues of investigation and gene knockouts through insertional mutagenesis are increasingly becoming available in ‘higher’ plants, which were previously only available in ‘lower’ plants, notably the moss Physcomitrella patens (Schaef er and Zryd, 1997). Antisense is obviously an important tool for identifying controlling steps in photosynthetic carbon assimilation, and thus for identifying new enzymes as targets for plant protection strategies (Hoefgen et al., 1995), making it an essential tool for applied research. For this reason it is possible that many antisense studies will only become public knowledge after patent issues have been settled.

The first study using antisense technology on Calvin cycle enzymes was that of Rodermel et al. (1988), who were able to reduce RbcS protein and mRNA levels in transgenic tobacco, and surprisingly found that this influenced the total amount of RbcL protein synthesized, but not the rbcL mRNA level. In a later study, they were able to show that this is because RbcS protein exerts a positive effect on the translation efficiency of rbcL mRNA in plastids, but not on the translation efficiency of other plastid mRNAs (Rodermel et al., 1996), providing insights into a longstanding problem of how nuclear and plastid gene expression might be coordinated. Many papers have appeared on antisense expression of...
The 3PGA pool remained quite stable, whereas the numbers of starch grains (Muschak et al., 1997). Yield under greenhouse conditions (Zrenner et al., 1996). Transgenic potato plants expressing antisense to as little as 7% of normal activity, and found that chloroplast FBPase showed reduced sizes and levels, but did not have negative effects on tuber light and temperature (Bilger et al., 1995; Fisahn et al., 1995). Reduction of cytosolic FBPase in potato light and temperature (Bilger et al., 1995; Fisahn et al., 1995; Jiang and Rodermel, 1995; Eckhardt et al., 1997). Varying degrees of reduced photosynthesis rates and various manifestations of altered redox state are usually observed in these plants. Furbank et al. (1994) studied the effect of rbcS antisense in a C4 plant and also found that, as in C3 plants, Rubisco activity was a major determinant of photosynthetic flux under high light intensities at elevated CO2.

Rubisco activase has also been inhibited by antisense. Plants required elevated CO2 to grow reasonably, Rubisco carbamylation was reduced and twofold increases in Rubisco levels were observed (Mate et al., 1993). In a separate study, reduction of Rubisco activase was not found to influence rbcS or rbcL mRNA or protein levels (Jiang et al., 1994). Reduction of Arabidopsis Rubisco activase to about 40% of the wildtype levels also produced plants that grow poorly, suggesting that Arabidopsis does not produce Rubisco activase in vast excess of its needs (Eckardt et al., 1997).

Reduction of chloroplast FBPase in potato to 15% of normal levels decreases tuber yield, but reduction to 36% did not effect yield, although photosynthesis was impaired (Kossmann et al., 1994). Chloroplast FBPase was reduced in tobacco and potato to roughly 20% of normal levels. These antisense mutants had impaired electron transport capacity, and plants kept at low temperatures and low light to reduce photoinhibition showed increased levels of deepoxidized xanthophylls in a manner that depended upon light and temperature (Bilger et al., 1995; Fisahn et al., 1995). Reduction of cytosolic FBPase in potato lowered sucrose biosynthesis and increased leaf starch levels, but did not have negative effects on tuber yield under greenhouse conditions (Zrenner et al., 1996). Transgenic potato plants expressing antisense chloroplast FBPase showed reduced sizes and numbers of starch grains (Muschak et al., 1997).

Price et al. (1995) measured metabolite pools in antisense tobacco with chloroplast GAPDH reduced to as little as 7% of normal activity, and found that the 3PGA pool remained quite stable, whereas the Ru1,5BP pool decreased as soon as GAPDH was below wildtype level. Reduction of PRK in tobacco to below 15% of normal levels resulted in up to five-fold increased levels of R5P, Ru5P, F6P and ATP, and up to 4-fold decreases in levels of Ru1,5BP, 3PGA and ADP. These metabolite levels largely compensated for PRK activity reduced down to 5% of the wildtype level, suggesting that it does not control flux through the pathway (Gray et al., 1995; Paul et al., 1995). Importantly, the extent of these effects depends in a crucial manner upon growth conditions, and most studies to date have been performed in controlled climate chambers under moderate light, rather than under variable ambient conditions including strong transients. Transgenic tobacco expressing antisense Rubisco activase showed reduced CO2 fixation rates and increased Rubisco levels (He et al., 1997). Antisense chloroplast aldolase effectively inhibited photosynthesis in potato (Haake et al., 1998). Reduction of PRK activity by 94% through antisense in tobacco had little effect on photosynthetic rate, except in the presence of low nitrogen (Banks et al., 1999).

It can be expected that antisense studies of the remaining Calvin cycle enzymes, clones for which are available from higher plants (Martin and Schnarrenberger, 1997) will eventually be reported. It is already becoming apparent that reducing the activity of TPI, PGK, TKL, RPE, RPI, which are not allosterically regulated in any particular manner, will more dramatically impair photosynthesis and growth (Stitt, 1999). However, it is also possible that no single enzyme limits total flux through the pathway, and that control may be distributed across various steps at which compensation can be exerted. Yet notably, very severe inhibition of photosynthesis was reported in tobacco expressing antisense SPBase, suggesting that this enzyme activity might exert strong control over the photosynthetic rate in this tissue (EP Harrison et al., 1998).

VIII. Concluding Remarks

The structural diversity known among Calvin cycle enzymes is great, but it is doubtful that its full breadth has been discovered. The numerous examples of class I and class II enzymes known from the pathway should prompt the question of just how difficult or unlikely it is for functionally (substrate-product) identical, but structurally (binding-catalysis)
distinct enzymes to evolve in nature de novo. Recent experiments suggest that it may not be as difficult as one might think: Wagner et al. (1995) produced antibodies that catalyze the class I aldolase reaction with a variety of ketones and aldehydes—some of which yield a $k_{\text{cat}}$ of $7 \times 10^{-3} \text{ min}^{-1}$ and a $K_m$ of 17 $\mu$M—these antibodies are true synthetic enzymes and employ the identical, Schiff-base mechanism as known from natural class I aldolase. If new enzymes can be generated in the lab in spans of years, then cells, given billions of years, should certainly be able to vastly surpass that result. Conversely, functional diversity can also emanate from one and the same structure, as shown by the biochemical data of Zhao et al. (1995), who found that erythrose-4-phosphate dehydrogenase of E. coli is encoded by a gene that was previously thought to code for a GAPDH. The latter principle may also be extended to the regulatory level, since the redox-regulatory motifs of one and the same enzymes can differ dramatically across species (Jacquot et al., 1997b). Uncovering the breadth of structural and functional diversity thus requires a balance of biochemical and molecular studies. Further investigation of the pathway in prokaryotes and photosynthetic protists should reveal new and unexpected examples of how sampling from ancient prokaryotic gene diversity can bring forth functionally equivalent, yet structurally divergent assemblies of Calvin cycle enzymes, pathways which, despite their origins from a maze of ancient diversity, bear—rightly so—a common unifying name.

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Rubisco: Assembly and Mechanism

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Summary

Ribulose-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39) is unique to photosynthetic metabolism. Two intensively studied aspects of Rubisco physiology are covered in this chapter, its post-translational assembly and its mechanism of action. Bacterial Rubisco can be assembled in vitro and in bacterial hosts but, as yet, assembly in vitro of higher-plant Rubiscos has not been reported. This focuses attention on the assembly pathway for higher plant Rubisco, which has been known for some time to be related to the presence of molecular chaperones in chloroplasts. Analysis of mutants, transformation of plants and bacteria with chloroplast chaperones, and the development of in vitro translation and assembly systems based on chloroplast extracts, have been directed at resolving this problem. It appears from these data that certain bacterial chaperones do not interfere with the assembly of higher plant Rubisco. As in cyanobacterial systems, the absence of S subunits leads to the accumulation of L- like particles whose subunits can later be recruited to form Rubisco. Subtle differences between the way S subunits assemble with higher-plant and cyanobacterial L- like particles suggest that this process may be concerted with assembly of L in the case of the higher-plant enzyme. The catalytic mechanism of Rubisco depends on two co-factors; a divalent metal ion, usually Mg\(^{2+}\), and a CO\(_2\) molecule that carbamylates a specific lysyl residue, K201, in the active site. This carbamate plays a crucial role in initiating catalysis by abstracting the C3 proton of ribulose bisphosphate and it may also act as a general-base catalyst for succeeding steps. So far, Rubisco’s use of a carbamate as a base appears to be unique among enzymes. The catalytic sequences of both the carboxylation reaction, and the oxygenation reaction that competes with it, proceed through multiple steps, each of a complexity rivaling that of the complete reaction of many other enzymes. The structure of the active site must change subtly between steps. Selectivity between \(\text{CO}_2\) and \(\text{O}_2\) of paramount importance to photosynthetic efficiency, is determined by the relative reactivity of the enediol(ate) form of the substrate for the two gases.

Abbreviations: 2\((or\ 4)\)-carboxyarabinitol-\(P\)\(_2\) \(\rightarrow\) 2\((or\ 4)\)-carboxyarabinitol-1,5-bisphosphate; carboxytetritol-\(P\)\(_2\) \(\rightarrow\) carboxytetritol-1,4-bisphosphate; Cpn60 \(\rightarrow\) chaperonin 60; pentodiulose-\(P\)\(_2\) \(\rightarrow\) \(D\)-glycero-2,3-pentodiulose-1,5-bisphosphate; \(P\)-glycerate \(\rightarrow\) 3-phospho-\(D\)-glycerate; \(P\)-glycolate \(\rightarrow\) 2-phosphoglycolate; ribulose-\(P\)\(_2\) \(\rightarrow\) \(D\)-ribulose-1,5-bisphosphate; Rubisco-ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39); xylulose-\(P\)\(_2\) \(\rightarrow\) \(D\)-xylulose-1,5-bisphosphate

I. Introduction

Many enzymes of photosynthetic metabolism are analogous to enzymes in other pathways and may have been recruited from them (Chapter 2, Martin et al.) but a few are known exclusively for their role in photosynthesis. Unquestionably, the most important
of these is the central CO₂-fixing enzyme ribulose-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39). Rubisco is believed to be the most abundant protein on earth (Ellis, 1979). Its catalytic effectiveness is quite feeble both in terms of its substrate-saturated \( k_{\text{cat}} \) (2–12 s⁻¹) and its \( k_{\text{cat}}/K_m(\text{CO}_2) \) ratio (5–40 \( \times 10^4 \) M⁻¹ s⁻¹) and this necessitates that photosynthetic organisms invest enormous amounts of protein in it (often approximately 50% of soluble leaf protein) to support acceptable rates of photosynthesis.

Rubisco catalyses both the carboxylation and the oxygenation of ribulose-\( \text{P}_2 \) within the same active site (Fig. 1). The carboxylation reaction, which produces two molecules of P-glycerate, is the \( \text{CO}_2 \)-acquiring reaction upon which the Calvin cycle (Chapter 2, Martin et al.) is based. The oxygenation reaction, which produces a molecule each of P-glycerate and P-glycolate, gives rise to photorespiratory metabolism (Chapter 5, Douce and Heldt). P-glycolate production results from Rubisco’s difficulty in discriminating between and \( \text{O}_2 \). The attendant photorespiration wastes carbon and light energy during photosynthesis.

Wildman and Bonner (1947) first discovered Rubisco as a major soluble protein detected by electrophoresis of extracts of plant leaves. Having no information about its function, they called it Fraction I protein. Early \(^{14}\text{CO}_2\)-labeling studies showing that P-glycerate was the first observed labeled product of photosynthesis pointed to the existence of an enzyme that catalyzed the carboxylation of ribulose-\( \text{P}_2 \) to produce two molecules of P-glycerate (Fig. 1) (Calvin and Massini, 1952; Quayle et al., 1954; Weissbach et al., 1954). Attribution of this enzymatic activity to Fraction 1 protein followed a decade later (Trown, 1965). Several more years were required before the second major activity of this protein, the oxygenation of ribulose-\( \text{P}_2 \) to produce a molecule each of P-glycolate and P-glycerate (Fig. 1) was discovered (Bowes et al., 1971; Andrews et al., 1973; Lorimer et al., 1973). Observation that the plant Rubisco was composed of two dissimilar kinds of subunits (Rutner and Lane, 1967) was followed by discoveries that the two subunits were encoded and synthesized in different cellular compartments (Blair and Ellis, 1973; Gooding et al., 1973; Smith et al., 1974; Roy et al., 1976). This led to the discovery of the mechanism for import of cytoplasmically synthesized proteins into chloroplasts (Chua and Schmidt, 1978). The plastid gene for the large, catalytic subunit was cloned and sequenced first from maize (McIntosh et al., 1980) and eventually from large numbers of other plants, algae and bacteria (Delwiche and Palmer, 1996; Watson and Tabita, 1997).

The physiology of Rubisco has been studied more than that of any other plant enzyme. In addition to the many properties described in other chapters in this volume, two important aspects of Rubisco physiology are covered separately in this chapter. Post-translational assembly of Rubisco has been a major focus of studies of chaperonin function, leading to insights into the folding of many large proteins. The mechanism of Rubisco catalysis has been studied by mutational analysis, X-ray crystallography, other biochemical techniques, and by analysis of the surprising number of side reactions that Rubisco catalyses. This chapter, then, covers the post-translational assembly and mechanism of action of Rubisco, current up to November 1998. The regulation
of Rubisco’s synthesis and activity are not covered here. These topics have been reviewed elsewhere in recent years (Hartman and Harpel, 1994; Andrews et al., 1995; Furbank and Taylor, 1995; Gutteridge and Gatenby, 1995; Portis, 1995; Salvucci and Ogren, 1996).

II. Rubisco’s Discordant Molecular Phylogeny

There are two major structural forms of Rubisco. Form I Rubisco, found in higher plants, algae, cyanobacteria, and many autotrophic bacteria, consists of eight ca. 53 kDa ‘large’ or ‘L’ subunits and eight ca. 14 kDa ‘small’ or ‘S’ subunits. Form II Rubisco, found in some dinoflagellates and certain autotrophic bacteria, including the obligate anaerobe, *Rhodospirillum rubrum*, lacks S subunits and contains just L subunits. The crystal structures of several Form I enzymes and the Form II enzyme from *R. rubrum* have been determined, enabling detailed mechanistic studies (see Section IV). In green algae and higher plants, Rubisco occurs in the chloroplast and the L subunit is encoded in the chloroplast genome. A family of nuclear genes encodes the S subunit, which is synthesized as a precursor polypeptide on cytosolic ribosomes and imported into the chloroplast in an ATP-dependent reaction that has been studied intensively (for a review, see Hartman and Harpel, 1994). In non-green algae, both L and S subunits are encoded in the chloroplast genome, confirming the hypothesis of differential migration of genes from the organelle genome to the nucleus that is a corollary of the endosymbiont theory of evolution (for a review, see Shivji et al., 1992). Rubisco L subunit genes of a large number of organisms have been sequenced but the phylogenetic tree resulting from analysis of 48 of these is not congruent with trees resulting from the analysis of rRNA and a variety of other proteins.

Two major classes of Form I Rubisco L subunit genes are distinguished: ‘green-like’ and ‘red-like,’ reflecting their predominant associations with plants, cyanobacteria and green algae on the one hand, and red algae on the other. However, both red-like and green-like Rubiscos are found among α, β and γ proteobacteria. In both red-like and green-like classes, two further sub-classes are apparent which again sometimes transgress rRNA-based phylogenies. Either there have been selective losses of some Rubisco genes in different branches of the phylogeny from an original ancestor that had all types of Rubisco genes or there have been multiple lateral transfers of Rubisco genes across taxonomic lines (Delwiche and Palmer, 1996).

III. The Folding and Assembly of Rubisco

A. Chaperones

Molecular chaperones are proteins that assist in the folding or assembly of other proteins but do not participate in their final structure (Netzer and Hartl, 1998). Examples are Hsp70 in *E. coli* or in the endoplasmic reticulum of eukaryotic cells (so-called BiP) and the GroEL and GroES proteins of *E. coli*. GroEL and GroES support the refolding in vitro of a large number of proteins from diverse sources, most notably the dimeric Rubisco from *Rhodospirillum rubrum* (for a review, see Gatenby, 1992). Proteins related in sequence to the *E. coli* GroEL and GroES proteins are called chaperoning (abbreviated Cpn60 and Cpn10, or in the case of the chloroplast, Cpn60 and Cpn21). The numerals in these abbreviations correspond approximately to the monomeric molecular weight of the polypeptides. The native state of Cpn60 is a tetradecamer consisting of two layers, each containing a ring of seven subunits. One Cpn10 ring binds to each Cpn60 ring (Hartl et al., 1994; Xu et al., 1997). Like the *E. coli* GroEL protein, the chloroplast Cpn60 is able to facilitate the refolding of dimeric *Rhodospirillum rubrum* Rubisco in vitro (Viitanen et al., 1995). The chloroplast Cpn21 homolog of Cpn10 has a similar structure, but the subunits are larger (Baneyx et al., 1995). Together with chloroplast Cpn60, it supports the refolding of mitochondrial malate dehydrogenase in vitro (Viitanen et al., 1995). It is clear from this that the chloroplast chaperonins indeed function as molecular chaperones. In vivo, it has been estimated from quantitative considerations that the chloroplast chaperonins are principally concerned with the synthesis and folding of the large subunit of chloroplast Rubisco (Lorimer, 1996). Kessler and Blobel (1996) reported that Cpn60 associates with IAP100, a component of the chloroplast protein import machinery, and that it can associate with imported mature Rubisco S subunits. This confirms an earlier report that Cpn60 can associate with imported chloroplast proteins (Lubben et al., 1989).
Chapter 3  Rubisco: Assembly and Mechanism

Apparently these turn over rapidly, because they do not represent an appreciable amount of radiolabeled material associated with Cpn60 in extracts derived from chloroplasts of pulse-labeled plants (Roy et al., 1982). The chloroplast Cpn 60 differs from that of E. coli in that it is encoded by two related genes rather than one (Hemmingsen et al., 1988). Also Na\(^+\) can replace K\(^+\) during refolding (Viiitanen et al., 1995) and the tetradecameric form of the chloroplast protein is much less stable in the presence of ATP (Hubbs et al., 1988). Thus, although there has been great progress in the analysis of prokaryotic chaperonin, it is likely that important aspects of the plant system can only be revealed by direct studies of the plant protein (for a recent review, see Boston et al., 1996).

We understand the chaperonin reaction mechanism in broad outline. Unfolded or partially folded polypeptides have a strong tendency to aggregate homotypically. They contain hydrophobic sequences that are exposed to the solvent. These proteins can bind to hydrophobic chaperonin surfaces that face toward the center of the heptameric rings of Cpn60 (Braig et al., 1994; Xu et al., 1997). After this, the details of the mechanism are controversial. One school of thought holds that the bound protein remains in the cavity formed by Cpn60 and Cpn 10, and undergoes a series of conformational shifts there (Ellis and Hartl, 1996; Martin and Hartl, 1997). Agents simulating the crowding effects of the high protein concentrations found in vivo appear to suppress the dissociation of folding proteins without inhibiting chaperone assisted folding (Martin and Hartl, 1997). Also, several proteins appear to fold completely while bound to mutant chaperonins that cannot form a double ring structure and thus do not dissociate in the normal manner (Rye et al., 1997).

Another view holds that in the presence of K\(^+\), MgATP and Cpn 10 cooperatively induce release of the bound polypeptide, which then can either aggregate, fold properly, or re-bind (Horwich et al., 1995; Burston et al., 1996; Török at el., 1996). If it aggregates, it may not fold; if it folds, it does not re-bind. If it re-binds, it has an indefinite number of opportunities to dissociate and rebind until it is either aggregated or folded. The net accumulation of folded polypeptide is often enhanced over that which occurs in the absence of chaperonins, in large part because aggregation is suppressed (Todd et al., 1994). However, there is sometimes a rate enhancement of folding, suggesting that the folding process itself is being catalyzed. One way to explain this is to suppose that protein folding occurs through a matrix of states of differing degrees of stability (Sfatos et al., 1996). Proteins arrested in an unfavorable configuration would be rescued if they could escape that configuration. The chaperonins may provide this opportunity by fostering actively the occurrence of conformational shifts (Todd et al., 1996). The idea is that, due to the energetics of nucleotide binding and hydrolysis, the chaperonin’s interior walls change shape, inducing conformational changes in the bound polypeptide in situ. In this model, a very critical part of the folding process (escape from an unfavorable configuration) is envisioned to take place while the protein is bound to the chaperonin complex, even if the protein is released and rebind before complete folding takes place.

B. Chaperonin-Mediated Folding and Assembly of Chloroplast Proteins

The E. coli chaperonins support the folding of the dimeric Rubisco of *Rhodospirillum rubrum*, either in vitro or in vivo (for reviews, see Roy, 1989, 1992; Ellis and van der Vies, 1991; Gatenby, 1992). The E. coli chaperonins also support the assembly of the hexadecameric cyanobacterial Rubisco in vivo (Ellis and van der Vies, 1991) but so far not in vitro. There is very strong evidence that the chloroplast Cpn60 normally participates in the assembly of hexadecameric higher plant Rubisco (discussed below). Chloroplast Cpn60 and Cpn21, and chloroplast Hsp70, have been found together to facilitate in vitro the reconstitution of the chloroplast coupling factor CFI, which has five different types of subunits (Chen and Jagendorf, 1994). They also support the folding of bacterial Rubisco and malate dehydrogenase (Viiitanen et al., 1995). In the latter study, Cpn21 was found not to be essential for refolding of the proteins, and Na\(^+\) could replace K\(^+\).

C. The Mechanism of Rubisco Assembly

*Rhodospirillum rubrum* Rubisco assembles as the result of a dimerization reaction that occurs very rapidly once the monomer is properly folded (for a review, see Hartman and Harpel, 1994). Hexadecameric Rubiscos from cyanobacteria dissociate into L\(_{\alpha}\) cores and free S subunits upon acid treatment. After neutralization, they can reassociate to form active enzyme (Andrews and Ballment, 1983). When cyanobacterial L subunits are cloned in *E. coli*, they
form either insoluble material or soluble L₈ core particles. In the latter case, S subunits can be added to reconstitute active enzyme. Alternately, the S subunits can be expressed in the same cell, resulting in the formation of active enzyme. Thus it is thought that after folding, the monomeric L subunits form dimers. These then aggregate to form the L₈ core, followed by binding of tetramers of S subunits to the top and bottom of the core. However, higher plant Rubisco subunits expressed in E. coli do not form active enzyme (for reviews, see Ellis and van der Vies, 1991; Gatenby, 1992; Roy, 1992). It is surprising that even when large subunits from higher plants bind to GroEL chaperonin, they fail to assemble properly. The reason for this is not understood.

In extracts of isolated pea chloroplasts, newly synthesized L subunits are found associated with chloroplast Cpn60. This association could in principle be a result of post-lysis binding of L subunits by Cpn60 (Lorimer, 1996). However, when chloroplasts pulse-labeled with ³⁵S-methionine are incubated in the light before lysis, there is a time-dependent decrease in the extent of binding of radioactive L subunits to Cpn60 in the extracts, which is not accompanied by any degradation of radioactive L subunits (Roy et al., 1988). Therefore, the binding must occur in the chloroplast before lysis. After synthesis and binding to Cpn60, Rubisco L subunits assemble into holoenzyme (Barraclough and Ellis, 1980). This assembly is itself dependent on continued post-translational illumination of the chloroplasts and can be detected within about 20 min after the onset of protein synthesis in the presence of radioactive amino acids (Roy et al., 1982). This result would not be expected if folding and assembly both were spontaneous processes. Alternately, if the chloroplasts are broken and the membranes removed, one can monitor the assembly reaction in vitro in the presence of MgATP, K⁺, and soluble S subunits (Milos and Roy, 1984; Roy and Gilson, 1996). The L subunits detach from Cpn60 and form binary complexes with each other or with Cpn60 monomers that dissociate from the Cpn60 tetradecamer. (The release of Cpn60 monomers does not seem to occur readily with E. coli Cpn60). The released L subunits can assemble into Rubisco in vitro. The assembly reaction itself is stimulated by ATP and can be inhibited specifically by antibody directed against chloroplast Cpn60 (Cannon, Wang, and Roy, 1986).

It is also possible to carry out translation of endogenous rbcL mRNA in chloroplast extracts, followed by Rubisco assembly in vitro. This reaction is completely dependent on added S subunits and is therefore more likely to be due to de novo assembly, rather than to subunit exchange (Hubbs and Roy, 1992). When protein synthesis was conducted over a range of temperatures in chloroplast extracts, followed by a constant temperature incubation, Rubisco assembly occurred only if translation had taken place at less than 32 °C (Hubbs and Roy, 1993a). Again, this result is not expected if protein folding is a spontaneous process with 100% yield. However, Rubisco synthesis in vivo occurs at higher temperature (Weidner and Fehling, 1985). Apparently disrupting the chloroplast dilutes all components or releases or activates proteases or interferes with post-translational processing (Houtz et al., 1992).

Hubbs and Roy (1993b) found that during Rubisco L subunit synthesis at low salt without small subunits, a previously undescribed oligomer accumulates. This oligomer shares electrophoretic and sedimentation properties with L₄ particles that have been detected in cloning experiments involving cyanobacterial Rubisco genes (Andrews, 1988; Goloubinoff et al., 1989a; Lee and Tabita, 1990). However, this oligomer does not bind S subunits at low KCl concentrations. At high KCl concentrations, it dissociates and the L subunits released then appear to assemble into holoenzyme. The results suggest that the Rubisco assembly mechanism in higher plants may be different from that expected based on work with cyanobacterial Rubisco, where it seems quite clear that stable L₄ cores bind S subunits to give rise to active enzyme in vitro (see Section IV.M.1). Could it be that L₄ cores are the products of side reactions, which occur to a greater extent in the absence of S subunits, and that the assembly of Rubisco normally proceeds through a variety of intermediates in the presence of S subunits? One likely intermediate in the assembly process is an L₂ dimer, based on the existence of form II Rubisco in prokaryotes, the presence of intimate L-L bonds in crystals of cyanobacterial and higher plant form I Rubisco, and on the presence of 7S (ca 110 kDa) L subunits in pea chloroplast extracts (Roy et al., 1982). The structure of unactivated tobacco Rubisco was described as four (LS)₂ dimers substantially stabilized by S subunits (Curmi et al., 1992). One of many possible assembly pathways then would be the formation of (LS)₄(LS)₄ structures, followed by aggregation of L₄S₄ units to reach the L₈S₈ structure.
D. Post-translational Modification of L Subunits

A number of post-translational modifications occur in the Rubisco large subunits of several higher plants, including pea (Houtz et al., 1992). These include trimethylation of lysine 14, and truncation and acetylation of the N terminus at proline 3. The enzyme catalyzing the trimethylation reaction has been cloned and sequenced (Klein and Houtz, 1995). Although there are some plants that do not carry out these modifications, it is possible that they play a role in assembly. Detailed molecular studies are needed to see whether this is the case.

E. Possible Involvement of Lipid-Protein Particles in Rubisco Assembly

Smith et al. (1997) reported that L subunits are associated with lipid-protein particles that are produced in light-dependent reactions by isolated thylakoids. No S subunits are associated with these particles, thus showing that these L subunits are not due simply to Rubisco holoenzyme sticking to the particles. Chloroplast chaperonin 60 subunits are also found associated with these lipid-protein particles. The authors suggested that the lipid protein particles might be coordinated with the assembly of Rubisco. However, no kinetic data concerning this hypothesis are available, so it is uncertain whether these L subunits are on the main pathway of assembly, a side pathway, or a degradative pathway.

F. A Model of Rubisco Assembly and the Limits of Our Understanding

A model incorporating most of the foregoing analysis is presented in Fig. 2. The model shows the unfolded L subunit, u-L, after having left the ribosome and any other unknown components with which it may have interacted, binding to the Cpn60-Cpn21 complex. In the presence of ATP, this complex equilibrates with unfolded L subunits and folded L subunits. Early immunological data (Cannon et al., 1986) indicated that it is possible that some of the L subunits associate with monomers of Cpn60. This type of intermediate has been suggested independently by studies of the effect of urea on the chaperonin-mediated refolding of rhodanese (Mendoza et al., 1994), by the observation that truncated monomers of Cpn60 can promote the folding of rhodanese (Makino et al., 1993), and very recently by the demonstration of chaperone activity by a monomeric unit of GroEL (Zahn et al., 1996). An alternate interpretation of the results of Cannon et al. (1986) is that even in the absence of ATP, unfolded L subunits can bind to and dissociate from Cpn60 in chloroplast extracts. If this is the case, then the L subunit pool seen in chloroplast extracts might consist principally of L subunit dimers and partly folded soluble monomers, also shown in the diagram. The soluble L₄-like oligomer, called Z in the diagram, can accumulate in the absence of S subunits but dissociates in high KCl concentration. The high-salt reactions may not be significant in vivo. However, the binding of S subunits to L subunits leads to the formation of holoenzyme. Although not shown, it is possible that S subunits also interact with the chaperone system, although they are not observed to accumulate under steady state conditions (Roy et al. 1982).

G. The Holy Grail of Rubisco Assembly

Despite the above evidence, so far nobody has demonstrated an absolute requirement for chloroplast Cpn60 in the assembly of higher plant Rubisco. Even when the genes for higher plant Rubisco are co-expressed in E. coli with the chloroplast Cpn60 genes, no assembly of Rubisco occurs (Cloney et al.,
nuclear-located chimeric gene containing the $RbcS$ transit sequence and tobacco $rbcL$ coding sequence. About 3% of normal Rubisco activity was observed in the transformed cells and the seedlings that they raised from these. These experiments show that the cytoplasmic environment permits cytosolic synthesis and transport of the modified $rbcL$ polypeptide to the chloroplast. It has been shown as well that a mutant tobacco, Sp25, harbors a mutation in the $rbcL$ gene that blocks Rubisco assembly (Shikanai et al., 1996). This mutant may be a suitable host for expression of modified Rubisco genes. Recently, Kanevski et al. (1999) replaced the $rbcL$ gene of tobacco chloroplasts with the analogous gene from sunflower and succeeded in forming a hybrid Rubisco with sunflower L subunits and tobacco S subunits in the resulting transgenic plants. Although the enzyme was not active enough to sustain photosynthetic growth, the authors were able to purify and characterize it. This achievement demonstrates the feasibility of chloroplast transformation for cloning and expressing genetically engineered Rubisco L subunits in higher plants.

All attempts at in vitro reconstitution of unfolded hexadecameric Rubisco from any source have failed (Lorimer, 1996). Fortunately, it is still possible to study the assembly of hexadecameric Rubisco in chloroplast extracts. Building on this may enable a more refined system to be developed.

### IV. The Catalytic Mechanism of Rubisco

#### A. Reversible Activation

Once the polypeptides are assembled correctly, Rubisco must be reversibly activated before catalysis can occur. Demonstrations that pre-incubation with $\text{CO}_2/\text{HCO}_3^-$ and $\text{Mg}^{2+}$ before assay in vitro increased activity (Pon et al., 1963; Andrews et al., 1975), that $\text{CO}_2/\text{HCO}_3^-$ became bound to the protein (Akoyunoglou and Calvin, 1963; Mizioro and Mildvan, 1974), and that activity and catalytic properties changed rapidly following extraction from chloroplasts and leaves (Badger and Andrews, 1974; Bahr and Jensen, 1974), led to the discovery that sequential binding of $\text{CO}_2$ and a divalent metal ion (physiologically $\text{Mg}^{2+}$) is a prerequisite for catalysis (Lorimer et al., 1976; Laing and Christeller, 1976). The oxygenase activity also requires this activation (Badger and Lorimer, 1976).
B. An Unusual Cofactor—\( \text{CO}_2 \)

1. Carbamylation Chemistry

The \( \text{CO}_2 \) molecule involved in the activation process, which binds in a reversible and rate limiting process before subsequent rapid binding of \( \text{Mg}^{2+} \) (Lorimer et al., 1976), is distinct from the substrate \( \text{CO}_2 \) molecule fixed during carboxylation (Lorimer, 1979). It becomes covalently attached as a carbamate to the \( \varepsilon \) amino group of a specific lysyl residue, K201\(^1\), within the active site (Lorimer, 1981). As discussed later (Section IV.F.2), this carbamate is now thought to play an instrumental role in catalysis and therefore qualifies as a cofactor in every sense of the word. Only after the metal-stabilized carbamate is in place is the active site able to convert ribulose-\( \text{P}_2 \) to products (Fig. 3).

2. Structure of the Carbamylated Active Site

Crystallographic studies have revealed the structural basis of the synergy between \( \text{CO}_2 \) and \( \text{Mg}^{2+} \) binding (Taylor and Andersson, 1996; Taylor and Andersson, 1997) (Fig. 4). The dispositions of the active site residues involved in carbamylation and \( \text{Mg}^{2+} \) binding are similar in the ER, ECM, ECMR and ECM-analog complexes. Carbamylation converts the side chain of K201 from a positive to a negative charge and the metal coordinates to one of the resulting carbamino oxygen atoms. Therefore, it is not surprising that carbamylation must occur before the metal can bind. Two protons are released during carbamylation (Fig. 3) and this is reflected in the pH response of carbamylation (Lorimer et al., 1976). The slowness of \( \text{CO}_2 \) addition relative to \( \text{Mg}^{2+} \) binding may be a result of scarcity of the unprotonated form of K201.

In addition to carbamylated K201, the protein provides two other ligands to the metal. These are carboxyl oxygen atoms of D203 and E204. In the

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\(^1\)The numbering of amino acid residues in both Rubisco subunits is based on that of the spinach enzyme. The alignments of Schneider et al. (1990) and Hudson et al. (1990) may be used to convert the residue numbers of the \( R. \text{rubrum} \) and \( \text{Synechococcus PCC} \text{6301} \) enzymes, respectively, to that of spinach. Amino acid residues are represented by their single-letter codes followed by the sequence number. A mutant residue is represented by the sequence number with the natural residue as a prefix and the mutant substitution as a suffix.
Fig. 4. Views of the active site of spinach Rubisco showing residues involved in catalysis and binding of the metal ion (stick rendering). a, ribulose-P₂ bound to the decarbamylated (inactive), metal-free site (ER) (Taylor and Andersson, 1997); b, the carbamylated, Mg²⁺-activated site without substrate (ECM) (Taylor and Andersson, 1996); c, the carbamylated site with Ca²⁺ and ribulose-P₂ bound (ECMR) (Taylor and Andersson, 1997); d, the carbamylated, Mg²⁺-activated site with 2'-carboxyarabinitol-P₂ bound (ECM-analog) (Andersson, 1996). Heavy atoms of the phosphorylated ligands, metal ions and water molecules are rendered as balls. Also see Color Plate 1.

absence of substrate, water molecules (Fig. 4b) occupy the other three metal-coordination positions. When ribulose-P₂ binds, two of these water molecules are displaced by the O-2 and O-3 atoms of the substrate (Fig. 4c). Ribulose-P₂ can also bind to the uncarbamylated, metal-free active site (Fig. 4a). This blocks the access of the metal to the active site, thus explaining observations that pre-exposure of Rubisco to ribulose-P₂ in the absence of metal seriously retards the carbamylation process (Jordan and Chollet, 1983). Since ribulose-P₂ binds to E three orders of magnitude more tightly than its $K_\text{m}$ for catalytic conversion by ECM (Jordan and Chollet, 1983), carbamylation is effectively prevented when ribulose-P₂ is present. In vivo, this impasse is broken by the action of Rubisco activase, which mediates the release of ribulose-P₂ from its dead-end complex with uncarbamylated Rubisco in an ATP-dependent reaction (for reviews, see Portis, 1992; Andrews et al., 1995; Portis, 1995; Salvucci and Ogren, 1996).

C. The Sequence of Catalytic Events

1. Substrates React in an Ordered Sequence

Once activated, with the lysyl carbamate and the metal in place, the stage is set for catalysis. The reaction is ordered with ribulose-P₂ binding before addition of the gaseous substrates, $\text{CO}_2$ or $\text{O}_2$. This is established by two complementary lines of evidence
First, NMR studies revealed no enhancement of the relaxation rate of $^{13}\text{CO}_2$ by Mn$^{2+}$ bound at the active site although similar evidence of binding was easy to observe with HCO$_3^-$, which binds loosely in the sites normally occupied by the phosphate groups of ribulose-$P_2$. Even when xylulose-$P_2$ was present, no CO$_2$ binding could be detected. Second, pre-exposure of Rubisco to $^{14}\text{CO}_2$ followed by simultaneous addition of ribulose-$P_2$ and an excess of unlabelled did not result in the production of labeled P-glycerate. Equilibrium binding experiments also showed no sign of O$_2$ binding in the absence of ribulose-$P_2$, even in the presence of xylulose-$P_2$. Taken together, these observations lead to the conclusion that no binding site for CO$_2$ or O$_2$ exists in the absence of ribulose-$P_2$, as would be expected if the gaseous substrate added first or if the order was random.

### 2. Sequence of Catalytic Intermediates

The catalytic processes of carboxylation and oxygenation involve a sequence of analogous intermediates except for a final protonation that is lacking in the case of oxygenation (Fig. 5). The preliminary enolization of ribulose-$P_2$ is common to both pathways. CO$_2$ or O$_2$ then compete for the resulting enediol$^2$ producing either a carboxyketone or a peroxyketone, respectively. These ketones are hydrated, either in concert with the addition of the gases or subsequently. The hydrated ketones then cleave heterolytically between C-2 and C-3. In the case of oxygenation, this completes the reaction. Carboxylation involves one further step: a proton must be added to the Si face of C-2 of the aci-acid produced from C-1 and C-2 of ribulose-$P_2$ and the incoming CO$_2$ molecule in order to produce the second molecule of P-glycerate.

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$^2$ During the course of the reaction, the enediol intermediate probably exists in three different protonation states depending on whether O2, O3 or both are protonated. It is also possible that the enediol is activated for electrophilic attack on C2 by acquiring some carbanionic character at this carbon. Unless otherwise specified, all of these states are referred to genetically as the 'enediol'.
D. Structural Transitions Associated with Ribulose-\(P_2\) and Analog Binding

1. Three-Dimensional Structure of Rubisco

Detailed three-dimensional structures have been determined crystallographically for the Form II (\(L_2\)) Rubisco of *Rhodospirillum rubrum* and the Form I (\(L_2S_8\)) Rubiscos of *Synechococcus* PCC6301 (a cyanobacterium), tobacco and spinach. In each case, structures have been determined with several different ligands occupying the active site. Some structures without ligand, and with or without the activating carbamate and metal ion, have also been determined. The available structures have been tabulated by (Cleland et al., 1998). Despite the considerable sequence divergence between the large subunits of Form I and Form II Rubiscos (approximately 25% identity clustered in the loop regions that contribute to the active site (Andrews and Lorimer, 1987)), the structures of the two forms are strikingly similar.

The large subunit consists of two main domains (Fig. 6). The N-terminal 150 residues form a five-stranded mixed \(\beta\) sheet with two \(\alpha\) helices on one side. The C-terminal domain is a classical eight-stranded \(\alpha/\beta\) barrel. This basic architecture is
maintained in all Rubiscos, both Form I and Form II. Like other $\alpha/\beta$ barrel enzymes, the active site is located at one end of the barrel, C-terminal with respect to the eight $\beta$ strands. Most of the residues involved in metal binding and catalysis occur in the loops that connect the $\beta$ strands to the helices. The others are located in the N-terminal domain of a companion L subunit, making $L_2$ the minimum functional unit. The $R. rubrum$ enzyme consists of this unit alone. Ligands bound at the active site are completely sequestered from solvent by several mobile loops that undergo a disorder-to-order transition as they close over the ligand (Schreuder et al., 1993).

The small subunits, present only in Form I Rubisco, are composed of a four-stranded anti-parallel $\beta$ sheet flanked on one side by two $\alpha$ helices (Fig. 6). They occur as $S_4$ clusters, one such cluster capping each pole of the $L_4$ octamer. They are quite remote from the active sites and this arrangement suggests that the $S_4$ clusters play a role in stabilizing the octameric core.

### 2. Binding to Uncarbamylated Site

Ribulose-$P_2$ and its analogs bind to both uncramylated and carbamylated active sites. When the site is not carbamylated, and therefore metal-free, the complex with ribulose-$P_2$ is unreactive and very stable. This has allowed the structure of this complex with spinach Rubisco to be determined crystallographically (Fig. 4a) (Taylor and Andersson, 1997). Similar structures are also seen with the analogs, xylulose-$P_2$ and 4′-carboxyarabinitol-$P_2$, with both *Synechococcus* and spinach Rubiscos (Newman and Gutteridge, 1994; Taylor et al., 1996). These structures are all similar and, except for the absence of the activating carbamate and the metal ion, similar to the structure of the complex of the carbamylated enzyme with 2′-carboxyarabinitol-$P_2$ (Section IV.D.3). In particular, the active sites in these structures are in a fully ordered, closed conformation with the ligand completely sequestered from solvent.

### 3. Binding to Carbamylated Site

Binding of 2′-carboxyarabinitol-$P_2$ to the carbamylated active site also induces closure, as shown by the structures of this complex with the *Synechococcus* (Newman and Gutteridge, 1993), tobacco (Schreuder et al., 1993) and spinach (Knight et al., 1990; Andersson, 1996) enzymes. All other structures show the active site in its open form. These include ligand-free structures with (Taylor and Andersson, 1996) or without (Curmi et al., 1992) the carbamate and metal, a carbamylated structure with two molecules of the product, P-glycerate, bound (Taylor and Andersson, 1997) and a complex between carbamylated spinach Rubisco and ribulose-$P_2$ rendered inactive by substitution of $Ca^{2+}$ for $Mg^{2+}$ (Taylor and Andersson, 1997).

### 4. Structural Changes Accompanying Closure

Structural differences between the open and closed conformations of the active site provide information about the transitions that must occur during every catalytic cycle (Schreuder et al., 1993; Taylor and Andersson, 1996). The most spectacular is the movement of loop 6 (residues 333–338) of the barrel domain. This region moves to cover the ligand like a hinged lid. The $\alpha$ carbons of the two residues at the apex of this lid move over 10 Å during this closure so that their side chains come into contact with the ligand. Residues 64–68 and the preceding helix of the N-terminal domain of the companion L subunit also move closer to the active site during ligand binding and residues 9–21 become ordered. At the C terminus, two turns of the C-terminal helix unwind so that the C-terminus can extend over loop 6, locking it in the closed position. In concert with these movements, the entire small subunit and the N-terminal domain of one LS pair pivot slightly with respect to the barrel domain.

### 5. When Does the Active Site Close?

The stage of the catalytic cycle (Fig. 5) at which this closure occurs is not presently known. The structural evidence establishes that it must occur by the time the carboxylated intermediate, 3-keto-2′-carboxyarabinitol-$P_2$, has formed and that it reopens for product release. A closed, desolvated active site would also be necessary to protect the enediol intermediate from incorrect protonation (Edmondson et al., 1990d). However, the open state of the activated complex with $Ca^{2+}$ and ribulose-$P_2$ presents a puzzle (Taylor and Andersson, 1997). Does this mean that active-site closure occurs during the act of enolization? Or is closure hindered by the larger size of $Ca^{2+}$ compared to the functional metals?
E. The Central Role of the Metal

Rubisco’s catalytic chemistry is centered on the metal, which pervasively influences all of the steps of the reaction. The 1.6 Å structure of the 2′-carboxyarabinitol-\(P_2\) complex with activated spinach Rubisco (Andersson, 1996) provides an excellent view of the way the metal is liganded (Fig. 4d). The octahedral coordination arrangement is somewhat distorted with rather long metal-ligand bond distances. Three of the ligands are provided by the protein and three by the analog, all of them O atoms. The four ligands in the equatorial plane (as defined by the shading in Fig. 7) are carboxyl O atoms of D203 and E204 and O2 and O3 of the analog. The ligand above the plane is provided by a carboxyl O atom of the analog; the one below the plane is a carbamino O atom of carbamylated K201. When the substrate analog is missing, water molecules substitute for its three ligands (Fig. 4b). The two water molecules in the equatorial plane are displaced by ribulose-\(P_2\) as it binds (Fig. 4c); the one above the plane is displaced by the incoming \(CO_2\) molecule (and presumably by the incoming \(O_2\) molecule in the case of oxygenation) as the carboxylated (or oxygenated) intermediate is formed.

F. The First Hurdle—Enolization

1. Occurs in the Absence of Gaseous Substrate

The first step of the catalytic sequence is the only one common to both carboxylation and oxygenation. It requires the removal of a proton from C3 of ribulose-\(P_2\) and addition of a proton to O2 (Figs. 5 and 7A,B). Protonation of O2 is essential. If this O atom were left unprotonated, gas addition in the next step would be directed towards C3, not C2. At some stage, deprotonation of O3 is also required to ensure gas addition to C2—see Section IV.F.4.

Enolization can be measured independently of overall catalysis by observing the exchange of the C3 proton with solvent when either the proton or the solvent is suitably labeled (e.g. see (Sue and Knowles, 1982; Gutteridge et al., 1984; Pierce et al., 1986; Lorimer and Hartman, 1988)). The exchange rate is highest when \(CO_2\) concentration is low and is suppressed at high \(CO_2\) concentration (Pierce et al., 1986), implying that enolization precedes \(CO_2\) addition and that reprotonation of the enediol and carboxylation of it are competing reactions.

2. Is the Carbamate the Essential Base?

Before the advent of crystallographic structural information about Rubisco’s active site, speculation abounded about the identity of the residue that initiated the catalytic sequence by abstracting the C3 proton of ribulose-\(P_2\). Several possibilities were advanced, based on circumstantial evidence. Early crystallographic evidence ruled out some of these notions but did not shed further light on the question because of inadequate resolution and confusion about which O atoms of the substrate were liganded to the metal. The confusion was finally resolved when the structures of the 2′-carboxyarabinitol-\(P_2\) complexes with the carbamylated Synechococcus (Newman and Gutteridge, 1993) and spinach (Andersson, 1996) enzymes were determined at high resolution. These established unequivocally that O2 and O3 were the metal-coordinated substrate O atoms and they showed that only one residue was appropriately positioned to act as the proton abstracter. Carbamylated K201 was monodentately coordinated to the metal via one of its carbamate O atoms; the other O atom of the carbamate seemed ideally positioned with respect to C3 of the analog to abstract the C3 proton of the substrate. This was confirmed in the structure of the spinach Rubisco/\(Ca^{2+}/ribulose-P_2\) complex where this O atom is 3.1 Å from C3 (Taylor and Andersson, 1997).

Despite this precise structural identification, uncertainty persisted about whether a carbamate O atom so positioned could be basic enough to remove a proton from carbon. Metal coordination of one O atom of a carboxylate, for example, lowers the \(pK_a\) of the other O atom so much that it can no longer act as a general base. However, a carbamate has access to a resonant structure where both O atoms are negatively charged and the N atom is positively charged. Proof that such an aci-carbamate can exist in proteins was provided by the structures of urease (Jabri et al., 1995) and phosphotriesterase (Benning et al., 1995). In both of these enzymes, a carbamate is monodentately coordinated to each of two divalent metal ions (Ni\(^{2+}\) or Zn\(^{2+}\)), indicating that both carbamate O atoms must bear substantial charge. In the case of Rubisco, a hydrogen bond between the carbamino N atom and the main-chain carbonyl O of residue 202 might assist in stabilizing the aci-carbamate (Lundqvist and Schneider, 1991).

Quantum chemical calculations were applied to the enolization step of the Rubisco reaction to assess whether it is likely that the carbamate could be the
Fig. 7. A proposed catalytic mechanism for the carboxylation of ribulose-P₂ showing the suggested roles of various active-site residues. Compared to Fig. 4, the structures are viewed rotated by approximately 90° (clockwise as viewed from P₁) around an axis through the two phosphorus atoms. During enolization (A [arrow] B), deprotonation of C3 by the Lys-201 carbamate must occur first but the timing of the subsequent protonation of O₂ and deprotonation of O₃ is not specified. Carboxylation of C2 of the enediol B is shown preceding hydration at C3 but a concerted mechanism without intermediate C is also plausible. Known side reactions of the carboxylation pathway are shown with dashed arrows.
proton abstracter. Using a 29-atom fragment model of the Mg2+-complexed active site in which glycolaldehyde represented C2 and C3 of the substrate, formate ions represented D203 and E204, and methyl carbamate represented carbamylated K201, states along a proposed enolization pathway were calculated (King et al., 1998). Calculations at several levels of theory yielded estimates of the energy of the transition state associated with transfer of the proton from C3 to the carbamate O atom that were low enough to lend credence to the notion that the carbamate could function in this way.

The basicity of the carbamate could be effectively increased in a way not modeled by the quantum chemical calculations. In the spinach Rubisco/Ca2+/ribulose-P2 complex, the non-coordinated O atom has access to a hydrogen-bonded network involving H327, Q401, S379, T173, two water molecules and the P1 phosphate group (Taylor and Andersson, 1997). This network could stabilize the protonated carbamate and allow the proton originally derived from C3 to exchange into it, consistent with the observed lack of retention of label derived from this proton during enolization.

3. Role of Lys-175 in Enolization

Another residue is also critical for enolization, K175. Its replacement with other residues caused 99.9% or greater inactivation of enolization and overall catalysis (Hartman et al., 1987; Hartman and Lee, 1989). Furthermore, the impairment appeared to be specific to the enolization partial reaction, the Gly-175 mutant enzyme being capable of binding 2'-carboxyarabinitol-P2 and cleaving the carbamylated intermediate, 3-keto-2'-carboxyarabinitol-P2 (Lorimer and Hartman, 1988). K175 also displays the unusually low pKa of 7.9 in its arylation by trinitrobenzenesulfonate (Hartman et al., 1985), a value close to that of an essential base observed in the pH-dependence of the deuterium isotope effect with [3-2H]ribulose-P2 (Van Dyk and Schloss, 1986).

Were it not for its totally inappropriate position and orientation (Figs. 4 and 7), all of this evidence might appear to establish K175 as the base that abstracts the C3 proton of ribulose-P2. Even with such a direct role ruled out, the data nevertheless show that K175 must have a very important role in enolization. What is this role? Participation in a proton relay that transports protons away from the active site has been hypothesized (Cleland et al., 1998). An alternative role envisages K175 acting as a general acid, not a base (Lundqvist and Schneider, 1991; King et al., 1998). While it certainly must act as a base in the reaction with trinitrobenzensulfonate, it does not necessarily follow that this must also be true for ribulose-P2 enolization. If K175 was protonated at the outset of the reaction (and its observed pKa of 7.9 is consistent with substantial protonation), then it would facilitate enolization first by assisting the metal to polarize the C2 carbonyl and second by protonating the developing negative charge on O2 (Fig. 7A,B), thus reducing the danger of misdirecting subsequent gas addition to C3. Quantum chemical calculations with the 29-atom representation of the active site, supplemented with either an ammonia molecule or an ammonium ion in the position occupied by the ε amino group of K175, showed that the presence of the ammonium ion (but not the ammonia molecule) stabilized the transition state for C3 proton abstraction by approximately 4 kcal mol−1. This value is quite consistent with the rate enhancement provided by Lys at position 175 compared to other residues. Furthermore, the protonation of O2 by the ammonium ion strongly stabilized the resultant enediol (King et al., 1998).

4. Deprotonation of O3

Protonation of O2 of the enediol is necessary to direct gas addition to C2 but not sufficient. Deprotonation of O3 is also required. H294 is appropriately positioned to accomplish this task (Figs. 4 and 7) and, in keeping with this assignment, its replacement with Asn or Gln virtually abolished catalysis (Harpe et al., 1998). However, these mutants were also disabled in enolization and in processing 3-keto-2'-carboxyarabinitol-P2, indicating that this residue influences several different catalytic steps. The near-total dependence of exchange of the C3 proton on H294 is a puzzle not readily explained by any current model of Rubisco’s catalytic mechanism. H294 is close to the carbamate and perhaps removal of the imidazole side chain disturbs the hydrogen-bonded network that stabilizes the protonated carbamate and facilitates the exchange of its proton (Section IV.F.2) (Harpe et al., 1998). Alternatively, if the hydrogen-bonded network can function to traffic the protonated carbamate’s proton away from the active site, then the carbamate itself would be available for re-use as a general base. It is also well positioned to deprotonate O3.
5. Stabilization of the Enediol Intermediate is not Completely Successful

The active site faces a challenging task in stabilizing such a highly reactive enolic nucleophile. It must protect it from attack by electrophiles other than CO₂ and frustrate the intrinsic tendency of enols to eliminate β-substituents.

**Attack by unwanted nucleophiles**—The most notable of these is O₂ which substitutes for CO₂ and causes the oxygenase reaction (Section IV.K). Stereochemically misdirected attack by protons presents another difficulty. Reprotonation of C3 from below the plane of Fig. 7 (i.e. from its Si face) reproduces the substrate, ribulose-P₃. Misdirected proton attack from above produces the epimer, xylulose-P₂. For the spinach enzyme, this occurs about once in every 400 turnovers and contributes to progressive inhibition during catalysis (Edmondson et al., 1990a,b,c,d). As expected, this misprotonation reaction is reversible, allowing xylulose-P₂ to serve as a very slowly reacting substrate (Yokota, 1991; Lee et al., 1993; Newman and Gutteridge, 1994). However, xylulose-P₂ eventually forces decarbamylation (Zhu and Jensen, 1991a). In vivo, inhibition by xylulose-P₂ presumably is reversed by Rubisco activase (for reviews, see Portis, 1992,1995; Andrews et al., 1995; Salvucci and Ogren, 1996). The wild-type *R. rubrum* enzyme, however, does not form appreciable amounts of xylulose-P₃ (Lee et al., 1993). Partitioning of product to xylulose-P₃, eventually forces decarbamylation (Zhu and Jensen, 1991a). The active site faces a challenging task in stabilizing such a highly reactive enolic nucleophile. It must protect it from attack by electrophiles other than CO₂ and frustrate the intrinsic tendency of enols to eliminate β-substituents.

However, xylulose-P₂ eventually forces decarbamylation (Zhu and Jensen, 1991a). In vivo, inhibition by xylulose-P₂ presumably is reversed by Rubisco activase (for reviews, see Portis, 1992, 1995; Andrews et al., 1995; Salvucci and Ogren, 1996). The wild-type *R. rubrum* enzyme, however, does not form appreciable amounts of xylulose-P₃ (Lee et al., 1993). Partitioning of product to xylulose-P₃ eventually forces decarbamylation (Zhu and Jensen, 1991a). However, xylulose-P₃ and O₂ become depleted (Morell et al., 1997).

Misprotonation at C2, rather than C3, is also theoretically possible. Proton attack from the Si face (the upper face in Fig. 7) would produce 3-keto-arabinitol-1,5-bisphosphate; from below it would produce 3-keto-ribitol-1,5-bisphosphate. Attack from above, the direction that CO₂ adds from, was speculated to occur (Edmondson et al., 1990d) and detection of arabinitol-1,5-bisphosphate after borohydride reduction of reaction products supported this speculation (Zhu and Jensen, 1991b; Morell et al., 1994, 1997; Zhu et al., 1998). However, under some conditions, arabinitol-1,5-bisphosphate can be produced nearly exclusively by reduction of a bisphosphorylated, dicarboxyl compound (pentodiulose-P₃) formed as an abortive by-product of the oxygenase reaction by mutants of *R. rubrum* Rubisco (Chen and Hartman, 1995; Harpel et al., 1995) and by non-enzymatic oxidation of ribulose-P₃ (Kane et al., 1998). This compound occurs as a contaminant in ribulose-P₂ preparations and it binds tightly to carbamylated Rubisco. It contributes substantially to the progressive inhibition observed during catalysis and is the dominant cause of this inhibition with poor quality ribulose-P₂ preparations (Kane et al., 1998). In view of this confusion, the actual extent of misprotonation of the enediol at C2 remains uncertain.

**β Elimination of the P₁ Phosphate**—Enols of phosphorylated sugars are very prone to loss of phosphate via β elimination reactions (Richard, 1984). Since these reactions involve elimination, not hydrolysis, desolvation within an active site provides no protection against them. Protection can be provided, however, by maintaining the bond to the β substituent in the same plane as the double bond, thereby minimizing orbital overlap between the substituent bond and the π system of the double bond (Rose, 1981). Although there is no structural information about the way the enediol is bound in the active site, the similarity between the structures of carbamylated spinach Rubisco with ribulose-P₂ and 2’-carboxyarabinitol-P₂ bound suggests that the enediol is bound in a similarly extended configuration. Furthermore, in the carbamylated Rubisco/Ca²⁺/ribulose-P₂ complex, O1, C1, C2 and C3 appear perfectly coplanar (Fig. 4c) (Taylor and Andersson, 1997). If this arrangement persists after formation of the enediol, β elimination would be minimized.

However, this stabilization strategy does not succeed completely. Production of 1-deoxy-D-glycero-2,3-pentodiulose-5-phosphate, the ultimate product of β elimination (Fig. 7B), can be detected with both spinach and *Synechococcus* Rubiscos following depletion of CO₂ and O₂ (Morell et al., 1997). Mutagenic perturbation of the P₁-binding site, which would be expected to impair ability to maintain the required planar configuration, drastically increases the proportion of the enediol β eliminated by both *Synechococcus* and *R. rubrum* Rubiscos (Larimer et al., 1994; Morell et al., 1994). Similar stimulation of the abortive reaction is also caused by replacement, in the *R. rubrum* enzyme, of K334 or deletion of the loop that contains it (Larson et al., 1995). This implies that blockage of carboxylation
of the enediol (Section IV.G.3) results in some of the enediol escaping from the active site to β eliminate in solution.

G. The Main Event—Gas Addition

1. No Michaelis Complex?

The correct tautomeric form of the enediolate (Fig. 7B) encourages CO\(_2\) (or O\(_2\)) addition at C2. As discussed in Section IV.C.1, the evidence seems convincing that neither gas binds in the absence of ribulose-P\(_2\). From this, it generally has been inferred that neither CO\(_2\) nor O\(_2\) forms a Michaelis (i.e. non-covalent) complex before reaction. This may well be true. It is difficult to imagine how such featureless molecules could bind non-covalently with substantial affinity. Nevertheless, the possibility of some sort of loose pre-reaction complex of the gases that forms only after ribulose-P\(_2\) has bound (or enolized) and the active site has closed is not yet formally excluded.

2. Is Addition of Gas and Water Concerted or Sequential?

Acid quenching of Rubisco during steady-state carboxylation releases the carboxylated intermediate, 3-keto-2- carboxyarabinitol-P\(_2\). It has a 1-h half life in neutral solution at 25 °C, decaying by decarboxylation, but can be trapped by borohydride reduction (Schloss and Lorimer, 1982; Lorimer et al., 1986). It exists in solution predominantly as the free carbonyl but on the active site it is fully hydrated (Pierce et al., 1986). When isolated 3-keto-2- carboxyarabinitol-P\(_2\) (free carbonyl) is supplied to Rubisco, it is processed to P-glycerate but at maximal rate of only one-fiftieth of the maximal rate of carboxylation of ribulose-P\(_2\) (Pierce et al., 1986). This might be interpreted as evidence that the free carbonyl form is not a normal intermediate of the reaction, i.e. that CO\(_2\) and OH\(^-\) addition to the enediol are concerted (Cleland, 1990). However, binding of 2- carboxyarabinitol-P\(_2\) (the analog of the gem-diol form 3-keto-2- carboxyarabinitol-P\(_2\) lacking one of the gem-diol hydroxyls) is a two-step process and the second, slower step proceeds at a rate comparable to the rate of turnover of 3-keto-2- carboxyarabinitol-P\(_2\) (Pierce et al., 1980; Pierce et al., 1986). Thus it is likely that processing of 3-keto-2- carboxyarabinitol-P\(_2\) is limited by a similar slow-binding step. Even so, this rate is still over 50-fold faster than the uncatalyzed hydration of 3-keto-2- carboxyarabinitol-P\(_2\) (Lorimer et al., 1986). Therefore we must conclude that Rubisco must bind the free carbonyl form of 3-keto-2- carboxyarabinitol-P\(_2\) and catalyze its hydration. The reduced rate of these processes compared to catalysis with the natural substrates may be caused by slower closure of the active site with the 2- carboxylate and is not necessarily evidence that the free carbonyl form does not lie on the catalytic pathway.

3. Features in the Active Site that Mediate CO\(_2\) Addition

Regardless of whether CO\(_2\) addition and hydration are concerted or sequential, three active-site features are required to facilitate the combined process.

First, a means of maintaining the negative charge on O3 is necessary to direct CO\(_2\) attack to C2. The interaction of this atom with the metal and with H294 (if it still bears the proton originally derived from O3) will achieve this. If the mechanism is sequential and the charge on O3 transiently disappears (Fig. 7C), the same features will be required to polarize the intermediate carbonyl to assist its hydration.

Second, the nascent carboxylate group at C2 must be stabilized. Structural (Fig. 4d) and mutagenesis and chemical-rescue (Lorimer et al., 1993; Larson et al., 1995) data concur in assigning the ε amino group of K334 a central role in this task in concert with the metal. Other side chains in the vicinity, such as Glu-60 (Lee et al., 1993) and L335 (Lee et al., 1993) also assist in maintaining accurate positioning of the K334 amino group.

Third, a general base is required to deprotonate the water molecule that attacks C3. Either H327 or H294 could serve in this way. If H294 is preoccupied accommodating the proton originally derived from O3, then the task might fall to H327. The crippling effects of mutating this side chain in Synechococcus Rubisco (Haining and McFadden, 1994) are consistent with such a role. On the other hand, substitution of residue 327 of R. rubrum Rubisco with Asn or Gln reduced both catalysis and enolization by only approximately 10-fold (Harpel et al., 1991), arguing against a total dependence on this residue alone. Again, the suspicion arises that the hydrogen-bonded network involving the K201 carbamate, H327,
Q401, S379, T173, two water molecules and the P1 phosphate group (Taylor and Andersson, 1997) collectively functions to absorb protons and transport them from the active site.

4. This Step is Effectively Irreversible

When 3-keto-2'-carboxyarabinitol-\(P_2\) is supplied as substrate to \(\text{Mg}^{2+}\)-activated Rubisco, it is converted nearly exclusively to P-glycerate. Little or no decarboxylation occurs, showing that the reaction is effectively irreversible once \(\text{CO}_2\) addition has occurred. If the metal is omitted, only decarboxylation is observed; intermediate partitioning between the forward and reverse pathways occurs when the enzyme is activated with \(\text{Mn}^{2+}\) or \(\text{Co}^{2+}\) (Pierce et al., 1986). Reverse partitioning is also seen with some mutants of \(R.\ rubrum\) Rubisco, e.g. K201E and H294N (Lorimer et al., 1987; Harpel et al., 1998). Interpretation of the significance of the decarboxylation reaction catalyzed by metal-free enzyme is clouded, however, by structural observations that the analog of the same intermediate, 2'-carboxyarabinitol-bound to the metal-free active site in reverse orientation, i.e. with the P2 phosphate group in the position normally occupied by P1 and vice versa (Lundqvist and Schneider, 1989; Zhang et al., 1994).

H. Carbon-Carbon Cleavage

1. The Facilitating Base

For cleavage between C2 and C3 to occur so that C3 is converted from a gem diol to a carboxylate, both of the gem-diol O atoms must be deprotonated. According to the mechanism formulated here (Fig. 7D), the metal-coordinated O atom is already deprotonated. Therefore, during C-C cleavage, the non-coordinated O also must lose its proton. This hydroxyl points towards the carbamate, which would be well positioned to acquire the proton (Fig. 7D) provided that it had been able to dispose of previously abstracted protons into its hydrogen-bonded network. For this to occur, the capacity of this network, and the associated histidyl residues 294 and 327, to absorb and/or transport protons must be quite prodigious. All four protons originally associated with C3 and the O atoms attached to it apparently are lost into this network.

2. The Aci-Acid Intermediate and its Stabilization

C-C cleavage produces one molecule of P-glycerate and the aci-acid (i.e. enol) form of a second (Fig. 7E). This intermediate has the same propensity to \(\beta\)-eliminate the phosphate group that the enediol intermediate has (Section IV.F.5). Presumably the same device stabilizes it; the C1-O bond to the phosphate must be held in the same plane as the carboxyl C-C2 bond (illustrated in Fig. 7E). However, a geometric conflict is now encountered. This is the same C1-O bond that, in the enediol intermediate, must be held co-planar with the C3-C2 bond which is perpendicular to the carboxyl C-C2 bond (compare Fig. 7B and Fig. 7E). To achieve the required planar configuration, either the C1-O bond or the carboxyl C-C2 bond (or both) must rotate on the C2-C1 axis through a total dihedral angle of 90° compared to their dispositions in the hydrated 3-keto-2'-carboxyarabinitol-\(P_2\) intermediate (compare Fig. 7D and Fig. 7E).

This critically important movement to co-planarity must occur within the constraints imposed by the coordination and hydrogen-bonded interactions which tether O2 to the metal, the carboxylate to the metal and to K334, and the phosphate to three Gly residues in loops 7 and 8 and to the T65 hydroxyl of the companion large subunit. These tethering interactions lie at opposite extremities of the aci-acid fragment, leaving C1 and C2 free to move. An economical clockwise movement of these two C atoms (as viewed from P1) about a line joining P1 and the metal would bring them into the required plane with O1 and O2 syn to each other. Such a movement involves only modest disturbance of the coordination with the metal of O2 and one carboxyl O and of the interaction of the other carboxyl O with K334. A small movement of O1 and a slight tilt of the plane through the three O atoms of P1 are also required. The structure of carbamylated spinach Rubisco complexed with two P-glycerate molecules reveals the upper P-glycerate molecule bound in a manner generally consistent with this interpretation, although in this complex loop 6 has retracted away from the active site and K334 no longer interacts with the carboxyl O atom (Taylor and Andersson, 1997).

As with the enediol intermediate, stabilization of the aci-acid is not completely successful. All wild-type Rubiscos studied abort their aci-acid inter-
mediate to produce pyruvate once in every 150 turnovers under a wide range of conditions (Fig. 7E) (Andrews and Kane, 1991). Again, this might be the result of inadequate stabilization of the aci-acid within the active site or escape of the intermediate into solution. Mutation of any of the residues that tether P1 perturbs this stoichiometry (Morell et al., 1994; Larimer et al., 1994). Replacement of the solvent with \( \text{H}_2\text{O} \) increases the tendency for the phosphate to be eliminated, presumably by slowing the subsequent protonation reaction (Andrews and Kane, 1991).

I. The Denouement—Stereospecific Protonation

1. Role of K175

To complete the reaction, protonation of C2 of the aci-acid intermediate, specifically on its Si face, is required. The stereochemical requirement is such that the proton donor cannot be any of the residues that have been involved in deprotonations earlier in the catalytic sequence (Fig. 7). Structural information about the identity of the donor is unambiguous. K175 is ideally placed. The aforementioned movement of C2 following C2-C3 cleavage brings it into optimal proximity to the amino N of K175 (Taylor and Andersson, 1997). Mutagenesis data are in perfect concordance. While K175 mutants of the \( R. \text{ rubrum} \) Rubisco retain ability to process 3-keto-2'-carboxyarabinitol- (Lorimer and Hartman, 1988), they do not produce the correct product. Pyruvate, instead of the upper P-glycerate molecule is a major product (Harpel and Hartman, 1996). Unable to acquire a proton quickly before the active site opens, the unstable aci-acid intermediate falls victim to \( \beta \) elimination.

K175 may not be the ultimate source of the proton supplied to C2. It has already donated a proton to O2 during enolization (Section IV.F.3). However, C2 is now very strongly basic and K175 may simply mediate the transfer of a proton from elsewhere to satisfy this basicity. For example, transfer of the O2 proton to C2, via K175, would be favorable thermodynamically. Such a close-coupled mechanism might be indifferent to solvent pH, in keeping with the observation that partitioning of product to pyruvate by spinach Rubisco is not influenced by pH between 6.4 and 9.1 (Andrews and Kane, 1991).

J. Product Dissociation

Breakage of the C2-C3 bond probably triggers opening of the active site for release of products. The structure of carbamylated Rubisco with two P-glycerate molecules bound (Taylor and Andersson, 1997) provides an excellent snapshot of this last stage of the catalytic cycle. These crystals were grown in the presence of a very high P-glycerate concentration (100 mM), forcing occupancy despite the poor affinity of the active site for products. The picture shows the active site open with upper P-glycerate in the expected position (as depicted in Fig. 7F). The lower P-glycerate is bound through its P2 phosphate but the rest of the molecule has swung out towards solvent.

K. The Oxygenase Reaction

The oxygenation reaction is presumed to involve a sequence of intermediates analogous to those of the carboxylase reaction. Presumably, in concert with C2-C3 cleavage, heterolytic cleavage of the peroxy O-O bond yields P-glycolate from C1 and C2 (Fig. 5). In keeping with this mechanism, one of the atoms of the \( \text{O}_2 \) molecule is incorporated into the carboxylate group of P-glycolate; the other is lost to solvent (Lorimer et al., 1973).

1. Spin Prohibited?

Formally, addition of ground-state, triplet \( \text{O}_2 \) to a singlet species such as the enediol is a spin-forbidden process. Furthermore, Rubisco lacks common devices for circumventing this barrier, such as redox-active metals or organic cofactors that facilitate radical chemistry. Despite this theoretical impediment, Rubisco manages to catalyze oxygenation of ribulose-P\(_2\) quite successfully and it is not unique in catalyzing \( \text{O}_2 \) addition to enols or carbanions (Abell and Schloss, 1991; Tse and Schloss, 1993; Hixon et al., 1996). Indeed, such additions are well known in organic chemistry. In Rubisco’s case, two mechanisms have been proposed for the \( \text{O}_2 \) addition step; both are purely conjectural.

2. Radical Mechanism?

A radical mechanism can be envisioned whereby two successive single-electron transfers occur from the C2 carbanion form of the enediol to \( \text{O}_2 \) (Andrews...
Chapter 3  
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and Lorimer, 1987). After the first transfer, a radical pair consisting of a superoxide anion radical and a C2 radical cation form of the enediol would exist in the active site. Interactions of these species with the metal and with the rest of the active site presumably would cage the pair long enough for spin inversion to occur and the peroxyketone intermediate to form.

3. Triplet Enediol?

Alternatively, it has been proposed that the carbanionic enediol has access to a low lying triplet state. Deformation of the enediol in the active site is hypothesized to reduce the energy gap between the singlet ground state and the first excited triplet state sufficiently that the approach of triplet O$_2$ induces intersystem crossing leading to a reactive supermolecule and eventually to singlet products (Andres et al., 1992; Tapia and Andrés, 1992; Andrés et al., 1993).

4. The Peroxyketone Intermediate

The carboxyketone intermediate of the carboxylase reaction can be isolated and used as a substrate and its structure in the active site can be inferred from that of its analog, 2-carboxyarabinitol-P$_2$. None of these advantages apply to the analogous intermediate in the oxygenase pathway and information about it is therefore largely inferential. Spectroscopic evidence obtained with Cu$^{2+}$-substituted spinach Rubisco favors an interaction of one of the O atoms of this intermediate with the metal (Brändén et al., 1984). If this is correct, then the interaction of the carboxylated intermediate with K334 must not be duplicated by the oxygenated intermediate. A role for this side chain in favoring carboxylation over oxygenation is therefore indicated, in accord with mutagenesis and chemical-rescue data for this residue (Gutteridge et al., 1993; Lorimer et al., 1993).

The most compelling evidence for the involvement of the peroxyketone intermediate comes from study of the E60Q and K334A mutants of the R. rubrum enzyme (Chen and Hartman, 1995; Harpel et al., 1995). These produce novel side products that are derived exclusively from the oxygenation pathway. The products are pentodiulose-P$_2$ and its product of benzylic acid-type rearrangement, carboxytetritol-P$_2$. Production of the former is accompanied by equimolar amounts of H$_2$O$_2$, indicating that it is derived by elimination of H$_2$O$_2$ from the peroxyketone intermediate. In the case of the K334A mutant, rearrangement of pentodiulose-P$_2$ to carboxytetritol-P$_2$ within the active site then ensues. This oxidative reaction also occurs non-enzymatically. Ribulose-P$_2$ is oxidized to pentodiulose-P$_2$ by molecular O$_2$ in a reaction catalyzed by transition metals (Kane et al., 1998). Unlike mutant or wild-type R. rubrum Rubiscos, spinach Rubisco does not convert pentodiulose-P$_2$ to carboxytetritol-P$_2$ or other products. Therefore, the carbamylated enzyme binds it in a very tight, dead-end complex that presumably mimics the complex with the enediol intermediate in the positions of all of the heavy atoms. The complex can, however, be disrupted easily with H$_2$O$_2$, yielding the normal oxygenase products, P-glycolate and P-glycerate (Kane et al., 1998). The importance of stabilizing the peroxyketone intermediate, suppressing H$_2$O$_2$ elimination from it, and encouraging its cleavage to P-glycolate and P-glycerate is thus underscored, particularly for the higher-plant enzyme. Better to catalyze a wasteful side reaction (P-glycolate production) than to sequester the enzyme in a dead-end complex with the H$_2$O$_2$-elimination product.

5. Chemiluminescence with Mn$^{2+}$

Spinach Rubisco activated by Mn$^{2+}$, but not by other metals, emits light while catalyzing oxygenation. This was initially interpreted in terms of the hypothetical radical mechanism of oxygenation (Section IV.K.2) and attributed to the simultaneous decay (the so-called dimol emission) of two molecules of singlet O$_2$ formed by reversal of the first single-electron transfer (Mogel and McFadden, 1990). However, Lilley et al. (1993) noted that the light emission was proportional to enzyme concentration and the rate of oxygenase catalysis and that it had a rather broad emission spectrum with a maximum at 770 nm. They considered it more likely that the light originated from an outer-orbital transition of the Mn$^{2+}$ ion excited by the presence of an excited species in its ligand field. Candidates for the exciting species might be the hypothetical radical pair of the radical model, the reactive supermolecule of the triplet model, or a species resulting from O-O cleavage of the peroxyketone. Chemiluminescence is also emitted from Mn$^{2+}$-activated R. rubrum Rubisco but with an intriguing difference: a pronounced burst of light emission occurs in the first second after ribulose-P$_2$ is added (Lilley et al., 1993). Perhaps this indicates that R. rubrum Rubisco is more limited by a late step
in its catalytic sequence (beyond the one causing light emission) than is the spinach enzyme. While mutation of S379 of *Synechococcus* Rubisco reduced oxygenation and chemiluminescence approximately commensurately (Lee and McFadden, 1992), the ratio between chemiluminescence and oxygenation varied drastically among several L335 mutations (Lee et al., 1993). Apparently, slight changes within the active site can greatly affect the luminescence efficiency.

**L. How is Selectivity between CO₂ and O₂ Achieved?**

Since the catalytic sequence is ordered with the gases reacting second in a mutually competitive manner, the relative specificity for CO₂ as opposed to O₂ is given by the ratio of the $V_{max} / K_m$ values for the two gases (i.e. $S_{eo} = (V_c / K_c) / (V_o / K_o)$ where $V_c$, $V_o$, $K_c$, and $K_o$ are the $V_{max}$ and $K_m$ values for the CO₂ and O₂, respectively (Laing et al., 1974)). $S_{eo}$ values for Mg²⁺-activated Rubisco at 25 °C vary over a 20-fold range (Table 1). Form II Rubiscos, which lack small subunits, have the lowest values, clustering around $10^{-15}$ except for the curious, nucleus-encoded Form II Rubisco from the dinoflagellate, *Amphidinium carterae*, which has a value approximately twice as high (Whitney and Andrews, 1998). Hexadecameric Form I Rubiscos have values ranging from a low of 35–40 in some bacteria and cyanobacteria up to a high in excess of 200 in some thermophilic rhodophytes. Higher plants cluster between 60 and 100.

As discussed in Section IV.G.1, it is likely that gas addition occurs without the aid of a Michaelis complex. Furthermore, once formed, the carboxyketone intermediate appears fully committed to product formation. The peroxycarbonyl intermediate is likely to be similarly committed, as it is in analogous organic reactions (Frankvoort, 1978). With the further assumption that both CO₂ and O₂ react with the same form of the enediol, it can be shown that the expression for $S_{eo}$ simplifies to the ratio of the second-order rate constants for addition of CO₂ and O₂ (Pierce et al., 1986; Lorimer et al., 1993). By this reasoning, the CO₂/O₂ specificity of a particular Rubisco is governed simply by the difference in reactivity of CO₂ and O₂ for the enediol. This is related to the difference in free energy between the transition states involved in the addition of the two gases (i.e. $\Delta G^{\ddagger}_{e-o} = RT \ln S_{eo}$, where $R$ is the gas constant, $T$ is the absolute temperature, and $\Delta G^{\ddagger}_{e-o}$ is the difference in free energy between the two transition states (Chen and Spreitzer, 1991; Lorimer et al., 1993). It thus becomes apparent that the differences in $S_{eo}$ between different Rubiscos are the result of very small differences in the relative stabilization of the transition states. The 7-fold difference in $S_{eo}$ between the higher-plant and *R. rubrum* enzymes, so crucial to the physiology of the organisms, amounts to no more than 1.2 kcal mol⁻¹ difference in relative free energy, approximately that contributed by a single hydrogen bond (Lorimer et al., 1993). An important caveat must be borne in mind when considering this analysis. It could become inappropriate if CO₂ and O₂ react with different species, such as the singlet and triplet states of the enediol discussed in Section IV.K.3. In this case, the interconversion between the two states would also influence the partitioning between carboxylation and oxygenation.

The CO₂/O₂ specificity is 10- to 25-fold greater when Mg²⁺ is the activating metal, compared to Mn²⁺ (Jordan and Ogren, 1983). The reduction caused by Mn²⁺ substitution is much greater than that predicted from the modest decrease in forward partitioning of the carboxyketone intermediate seen with Mn²⁺ (Pierce et al., 1986) and must reflect the role of the metal in stabilizing the transition states leading to carboxylation and oxygenation.

Apart from the obvious role of the K334 side chain in promoting carboxylation (Section IV.K.4), numerous other mutations in *R. rubrum*, *Synechococcus* and *Chlamydomonas reinhardtii* Rubiscos lead to reductions in $S_{eo}$ (Spreitzer, 1993) and (Wildner et al., 1996) have tabulated many of these. There are also a few examples of mutations leading to slight increases in this parameter, sometimes but not always at the cost of reductions in $V_c$ (Harpel and Hartman, 1992; Parry et al., 1992; Gutteridge et al., 1993; Kostov et al., 1997; Madgwick et al., 1998). Some of these changes can be rationalized in terms of indirect effects that the mutations may have on the crucial positioning of the ε amino N atom of K334. Effects of substitutions in loop 6 are usually so explained. For many others the connection seems quite indirect. Apparently, the slightest perturbation can be transmitted through the complex web of interactions in the active site to alter this crucial selectivity.
The great majority of Rubiscos in nature are hexadecameric proteins containing small subunits (Form I). All eukaryotic Rubiscos, except some from dinoflagellates (Morse et al., 1995; Rowan et al., 1996), fall into this class. However, the existence of bacterial and dinoflagellate Rubiscos that lack small subunits (Form II) shows that they are dispensable, at least in some circumstances. The primary sequences of the large subunits of Form II enzymes, forming a distinct group deeply divided from the rest of the large-subunit phylogeny (Watson and Tabita, 1997).

### Table 1. $S_{cn}$ values at 25 °C for Rubiscos from various sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Form</th>
<th>$S_{cn}$ (Mg$^{2-}$)</th>
<th>$S_{cn}$ (Mn$^{2-}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_3$ plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Spinacia oleracea</em></td>
<td>I</td>
<td>80</td>
<td>3.1</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Uemura et al. (1997)</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>I</td>
<td>77</td>
<td>3.2</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>I</td>
<td>82</td>
<td>3.9</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td><em>Lolium perenne</em></td>
<td>I</td>
<td>80</td>
<td></td>
<td>Jordan and Ogren (1981)</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>I</td>
<td>90</td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>I</td>
<td>87</td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td><em>Oryza punctata</em></td>
<td>I</td>
<td>85</td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td>C$_4$ plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Amaranthus hybridus</em></td>
<td>I</td>
<td>82</td>
<td>3.5</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>I</td>
<td>78</td>
<td>3.3</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td><em>Setaria italica</em></td>
<td>I</td>
<td>79</td>
<td></td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td>Green algae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>I</td>
<td>61</td>
<td>2.4</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
<td>I</td>
<td>63</td>
<td></td>
<td>Jordan and Ogren (1981)</td>
</tr>
<tr>
<td><em>Coccomyxa sp.</em></td>
<td>I</td>
<td>83</td>
<td></td>
<td>Palmeqvist et al. (1995)</td>
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<tr>
<td><em>Buglossis gracilis</em></td>
<td>I</td>
<td>54</td>
<td>2.8</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td>Non-green eukaryotic algae</td>
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<td></td>
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<td></td>
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<td><em>Porphyridium purpureum</em></td>
<td>I</td>
<td>144</td>
<td></td>
<td>Uemura et al. (1997)</td>
</tr>
<tr>
<td><em>Cyanidium caldarium</em></td>
<td>I</td>
<td>225</td>
<td></td>
<td>Uemura et al. (1997)</td>
</tr>
<tr>
<td><em>Galdiera partita</em></td>
<td>I</td>
<td>238</td>
<td></td>
<td>Uemura et al. (1997)</td>
</tr>
<tr>
<td><em>Amphidinium carterae</em></td>
<td>II</td>
<td>37 (10°C)</td>
<td></td>
<td>Whitney and Andrews (1998)</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Coccolithus peniocystis</em></td>
<td>I</td>
<td>47</td>
<td></td>
<td>Jordan and Ogren (1981)</td>
</tr>
<tr>
<td><em>Aphanizomenon flos-aquae</em></td>
<td>I</td>
<td>48</td>
<td>2.0</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td><em>Synechococcus PCC6301</em></td>
<td>I</td>
<td>43</td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>I</td>
<td>44</td>
<td></td>
<td>Uemura et al. (1997)</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>II</td>
<td>9</td>
<td></td>
<td>Jordan and Ogren (1981)</td>
</tr>
<tr>
<td><em>Rhodosporillum rubrum</em></td>
<td>II</td>
<td>15</td>
<td>1.5</td>
<td>Jordan and Ogren (1983)</td>
</tr>
<tr>
<td><em>Thiobacillus denitrificans</em></td>
<td>II</td>
<td>12</td>
<td></td>
<td>Kane et al. (1994)</td>
</tr>
</tbody>
</table>

$^a$ Form I Rubiscos are further classified according to whether they fall in the ‘green-like’ or ‘red-like’ phylogenies (see Section II).
1. In Promoting Catalysis

Early studies exploited the ability to reversibly dissociate the small subunits from the \( L_8 \) core of some cyanobacterial and bacterial Rubiscos (Andrews and Abel, 1981; Andrews and Bailment, 1983; Incharoensakdi et al., 1985; Jordan and Chollet, 1985). These revealed that all but a trace of activity was lost on removal of small subunits and that partially depleted preparations had activities commensurate with their small-subunit content. Expression of the \( rbcL \) gene from \( Synechococcus \) PCC6301 in \( E. coli \) without the companion \( rbcS \) gene confirmed that the \( L_8 \) core retains approximately 1% of the holoenzyme’s activity and that it can be fully activated in vitro if supplied with isolated small subunits (Andrews, 1988; Paul et al., 1991). Heterologous small subunits, even those from spinach Rubisco, partially restore activity (Andrews et al., 1984; Andrews and Lorimer, 1985). The \( L_8 \) core is competent in carbamylation and binds divalent metal ions and 2- \(-\)carboxyarabinitol-\( P_2 \) (Andrews and Bailment, 1984; Jordan and Chollet, 1985; Andrews et al., 1986; Andrews, 1988). Its competence in enolizing ribulose-\( P_2 \) and in processing 3-keto-2- \(-\)carboxyarabinitol-\( P_4 \) matches its limited competence in overall carboxylation, indicating that the impairment is shared between the various steps of the catalytic sequence (Andrews et al., 1986). The influence of the small subunit on the active site’s ability to suppress the various side reactions (Fig. 7) is more specific, however. In the absence of small subunits, some 10% of the enediol intermediate eliminates phosphate, rather than being carboxylated, but neither misprotonation of the enediol nor the pyruvate-producing side reaction are exacerbated (Morell et al., 1997).

2. In Determining CO\(_2\)/O\(_2\) Specificity

As discussed in Section IV.L, Rubiscos with small subunits discriminate against the oxygenation reaction better than those without. While this might imply a role for the small subunits in determining CO\(_2\)/O\(_2\) specificity, initial evidence was to the contrary. The \( L_8 \) core of \( Synechococcus \) Rubisco retains the same \( S_{e0} \) as its holoenzyme (Gutteridge, 1991), as does its hybrids with spinach (Andrews and Lorimer, 1985) and \( Alcaligenes eutrophus \) (Lee et al., 1991) small subunits. In contrast, hybridization of \( Synechococcus \) \( L_8 \) with small subunits derived from the diatom \( Cylindrotheca \) sp. N1, whose Rubisco has a very high \( S_{e0} \) (Table 1), results in a hybrid Rubisco with an \( S_{e0} \) value intermediate between those of the two parent holoenzymes (Read and Tabita, 1992). There is also a report of small-subunit mutations of the \( Synechococcus \) holoenzyme that alter \( S_{e0} \) (Kostov et al., 1997). Therefore, the small subunit obviously can have some influence on \( S_{e0} \) in some instances.

3. In Structure

Despite the integrity of the \( L_8 \) core of some cyanobacterial and bacterial Rubiscos in the absence of small subunits, the positions of the small subunits in the structure of the hexadecamer strongly suggest that they have an adhesive role in holding the four dimers of large subunits together. Each small subunit in the \( S_2 \) unit which caps each pole of the \( L_8 \) core interacts with two of its neighbors and, lying in a crevice between two \( L_2 \) units, also interacts with three different large subunits (Fig. 6A) (Knight et al., 1990). However, it is clear that the small subunit must have more than a simple adhesive role. Not only does it induce a 100-fold stimulation of catalysis and alter catalytic parameters such as \( S_{e0} \), but it also makes the equilibrium of the carbamylation reactions more favorable (Andrews and Bailment, 1984). There is also evidence of synergism between binding of small subunits and binding of 2- \(-\)carboxyarabinitol-\( P_2 \) (Andrews, 1988). Binding of small subunits must cause structural changes that are communicated to the active site. One possibility, inferred from a comparison of the structures of the spinach and \( R. rubrum \) enzymes (Schneider et al., 1990), is that contact between a small subunit and helix 8 of the barrel domain of the large subunit induces a change in loop 8 which contributes to the P1 binding site. The stimulation of \( \beta \) elimination of the enediol observed in the absence of small subunits (Morell et al., 1997) accords with this proposal. So too do observations that mutations and truncations of the conserved N-terminal region of the small subunit that contacts loop 8 impair catalysis, loosen inter-subunit binding, and alter \( S_{e0} \) (Paul et al., 1991; Paul et al., 1993; Kostov et al., 1997). However, the small subunit has several other conserved regions that contact different parts of the large subunit and mutations in these regions also impair catalysis (Voordouw et al., 1987; Lee et al., 1991; Smrcka et al., 1991; Read and Tabita, 1992) and exacerbate xylulose-\( P_2 \) production (Flachmann et al., 1997). All such contacts have the potential to transmit structural
information to the active site. Determination of the structure of the L$_4$ core without small subunits would provide useful further information about structural changes induced by the small subunits.

While structural changes induced in the active site by remote binding of small subunits provide a functional explanation for the role of the small subunit, this does not satisfactorily explain the existence of the small subunits. Form II Rubiscos function adequately without small subunits but have not achieved CO$_2$/O$_2$ specificities comparable to those of Form I enzymes. Reasons for the requirement of a separate polypeptide to impart the additional structural information necessary to attain greater specificity remain elusive.

V. Conclusion

The mechanisms of assembly of and catalysis by Rubisco are both characterized by daunting complexity. Despite the wealth of knowledge that exists about both aspects, no eukaryotic Rubisco has yet been assembled successfully in vitro or in any foreign host and the efforts to engineer improvements in catalytic efficiency have had very limited success. While more is now known about Rubisco than any other plant protein, this knowledge provides only incomplete understanding of critical aspects of both assembly and catalysis. Nevertheless, current Rubisco research represents an outstanding synthesis of modern chemical, molecular and structural biology. The objective of understanding Rubisco’s assembly and catalysis in sufficient depth to manipulate them presents a tantalizing challenge.

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Chapter 4

Rubisco: Physiology in Vivo

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A broad overview of the physiology and biochemistry of Rubisco is presented with a comparison of information obtained in vitro and in vivo. First a brief background to kinetic properties of Rubisco is given and Rubisco’s influence on photosynthetic metabolism is reviewed for C₃, C₄, CAM and C₃-C₄ species. The effect of environmental variables such as light and CO₂ are considered for both short and longer term effects on the activity and abundance of Rubisco protein. Over the past few years experiments with transgenic plants with antisense RNA constructs to Rubisco, Rubisco activase and several of the PCR cycle enzymes have added new insights into Rubisco physiology. For example, transgenic tobacco with reduced amount of Rubisco has allowed the identification of environmental conditions where Rubisco exerts maximal control on photosynthesis. These plants have also been used to determine Rubisco kinetic constants in vivo. Transgenic plants with reduced amounts of Rubisco activase have been used to elucidate the role of activase in vivo.

I. Introduction

In C₃ plants, Rubisco is at the gateway of photosynthesis facilitating the primary carboxylation of atmospheric CO₂. Its role in the photosynthetic carbon reduction (PCR) cycle and its catalytic mechanism have been discussed in previous chapters. The aim of this chapter is to provide a broad overview of the physiology of Rubisco in vivo as evident in the CO₂ fixation characteristics of leaves. There are already many excellent reviews discussing this topic such as those by Andrews and Lorimer (1987), Woodrow and Berry (1988), Sharkey (1989), Salvucci (1989) and Portis (1990). Over the past few years experiments with transgenic plants with antisense RNA constructs to Rubisco, Rubisco activase and several of the PCR cycle enzymes have added new insights (Stitt and Schulze, 1994).

In higher plants, the holoenzyme Rubisco is a hexadecamer consisting of eight large and small subunits with eight catalytic sites. To function, Rubisco’s catalytic sites must be carbamylated to allow the binding of a catalytically essential Mg²⁺ (for review see Andrews and Lorimer, 1987) and nocturnal inhibitors such as 2’-carboxyarabinitol 1-phosphate (CA1P) need also be removed from catalytic sites (Vu et al., 1984; Seemann et al., 1985). In most instances, Rubisco is fully carbamylated under high light. We will therefore first explore the relationship between CO₂ assimilation rate and fully active Rubisco and then consider the in vivo regulation of the activation process. To aid our discussion we use the photosynthetic model of Farquhar et al. (1980) to provide the quantitative link between measurements of CO₂ assimilation rate and Rubisco carboxylation rate.

II. Rubisco and CO₂ Assimilation Rate, a Quantitative Relationship

A. The Model

Here we give a brief description of the key equations of the model by Farquhar et al. (1980). A more detailed discussion of many of the individual parameters follows in later sections.

The CO₂ assimilation rate of leaves with the C₃ photosynthetic pathway is the net result of Rubisco carboxylations and CO₂ evolved during photorespiration and other mitochondrial respiration. The realization that the oxygen sensitivity of CO₂ assimilation could be explained by Rubisco oxygenase activity highlighted the role Rubisco plays in determining the net rate of CO₂ assimilation (Bowes
et al., 1971; Laing et al., 1974). We assume that in the photorespiratory carbon oxidation (PCO) cycle, oxygenation of one mole of RuBP leads to the release of 0.5 mole of CO₂ and the net rate of CO₂ assimilation, A, can thus be given by

\[ A = V_c - 0.5V_o - R_d, \]  

where \( V_c \) and \( V_o \) are the rates of Rubisco carboxylation and oxygenation, respectively, and \( R_d \) denotes mitochondrial respiration in the light other than that associated with the PCO cycle (Farquhar et al., 1980; Farquhar and von Caemmerer, 1982). Since CO₂ and O₂ are competitive alternate substrates of Rubisco (Laing et al., 1974; Chapter 3, Roy and Andrews) it follows that

\[ S_{C/O} = \frac{V_cO}{V_cC_c} = \frac{V_{\text{max}}K_o}{V_{\text{max}}K_c} \]  

where \( S_{C/O} \) is the relative specificity of Rubisco for CO₂ as opposed to O₂. \( C_c \) and \( O \) are the partial pressures of CO₂ and O₂ at the catalytic site, \( V_{\text{max}} \) and \( V_{\text{max}} \) are the maximal rates of carboxylation and oxygenation, and \( K_c \) and \( K_o \) are the Michaelis-Menten constants for CO₂ and O₂, respectively.

The CO₂ partial pressure at which the carboxylation rate equals the rate of photorespiratory release has been denoted \( \Gamma* \) (Laisk, 1977). When the partial pressure of CO₂ in the chloroplast, \( C_c \), is at \( \Gamma* \), then \( A = -R_d \) and \( V_c/V_o = 0.5 \). Therefore

\[ \Gamma* = \frac{0.5O}{S_{C/O}} \]  

and at any \( C_c \), the rate of Rubisco oxygenation can be related to the rate of carboxylation by

\[ V_o = -A \frac{\Gamma*}{C_c} \]  

and CO₂ assimilation rate can be expressed as a function of \( V_o, \Gamma* \), and \( C_c \):

\[ A = (1 - \Gamma*/C_c)V_c - R_d. \]  

Farquhar et al. (1980) showed that CO₂ assimilation rate can be given as either a RuBP-saturated rate or RuBP regeneration limited rate. This is because Rubisco is present at very high concentrations in the chloroplast (up to 4 mM) and the \( K_m \) for RuBP is quite low (20 \( \mu \)M, Badger and Collatz, 1977). The RuBP-saturated carboxylation rate of Rubisco is given by

\[ V_c = \frac{C_cV_{\text{max}}}{C_c + K_c(1 + O/K_o)}, \]  

which is the general rate equation for an enzyme with two competing substrates (Fersht, 1984). By substituting Eq. (6) into Eq. (5), CO₂ assimilation rate can be given as a function of chloroplastic CO₂ and O₂ partial pressures and Rubisco kinetic parameters

\[ A = \frac{(C_c - \Gamma*)V_{\text{max}}}{C_c + K_c(1 + O/K_o)} - R_d. \]  

To regenerate one mol of RuBP, two mol of NADPH are required in both the PCR and PCO cycle, which in turn requires two mol of electrons per NADPH. The electron transport rate, \( J \), satisfying the rate of NADPH consumption in the PCR and PCO cycle is therefore:

\[ J = 4(V_c + V_o) = 4(1 + 2\Gamma*/C_o)V_c \]  

(Farquhar et al., 1980). Thus when the rate of RuBP regeneration is limited by electron transport Farquhar et al. (1980) derived an equation for the electron transport (or RuBP regeneration) limited rate by combining Eqs. (5) and (8).

\[ A = \frac{(C_c - \Gamma*)J}{(4C_c + 8\Gamma*)} - R_d. \]  

Eqs. (7) and (9) are the two equations most important to our analysis. Figure 1 shows the modeled dependence of CO₂ assimilation rate on chloroplastic CO₂ partial pressures. The Rubisco-limited (or RuBP-saturated) rate is linearly dependent on \( V_{\text{max}} \), the maximal Rubisco rate. When \( A \) is limited by the rate of RuBP regeneration, \( \Gamma* \) determines the partitioning of available energy between carboxylation and oxygenation.

### B. Rubisco Specificity and the CO₂ Compensation Point

Rubisco’s relative specificity for CO₂ as opposed to O₂ (Eq. 2) is simply the ratio between the specificity for CO₂ \( (V_{\text{max}}/K_c) \) and the specificity for O₂ \( (V_{\text{max}}/K_o) \). \( S_{C/O} \) is often used as a performance index of Rubisco.

\[ S_{C/O} = \frac{V_c}{V_o}. \]
and has frequently been measured in vitro on purified Rubisco (Jordan and Ogren, 1981; Parry et al., 1987; Gutteridge, 1990; Kane et al., 1994). $S_{o_{i}}$ of Rubisco varies between taxa. It is lowest in photosynthetic bacteria (10-20 MM$^{-1}$, or 268-536 bar bar$^{-1}$) and intermediate in cyanobacteria ($\sim$48 MM$^{-1}$ or 1286 bar bar$^{-1}$) and green algae ($\sim$60 MM$^{-1}$ or 1608 bar bar$^{-1}$), and is greatest in higher plants (MM$^{-1}$ 80–100, or 2144-2680 bar bar$^{-1}$) (Jordan and Ogren, 1981). Careful in vitro measurements of $S_{o_{i}}$ at 25 °C have shown only minor variation amongst C$_{1}$ species (Kane et al., 1994). The higher plant numbers state that at equal dissolved concentrations of O$_{2}$ and CO$_{2}$ the carboxylation rate would be 80 to 100 times the rate of oxygenation (Eq. (2)). At equal partial pressures of O$_{2}$ and CO$_{2}$ in the gas phase the carboxylation rate is 2000 to 3000 times the oxygenation rate because the solubility of O$_{2}$ is much less than that of CO$_{2}$ (Table 1).

The CO$_{2}$ compensation point, $\Gamma$ (the CO$_{2}$ partial pressure at which there is no net CO$_{2}$ assimilation rate) provides the link between the kinetic properties of Rubisco and the gas exchange of leaves. In the absence of any day respiration, $R_{d}$, $\Gamma = \Gamma_{c}$, and is thus directly proportional to the inverse of $S_{o_{i}}$ (Eq. (2)); in the presence of respiratory activity

$$\Gamma = \frac{\Gamma_{c} + Kc (1 + O/Ko) R_{d}}{I - R_{d}/V_{cmax}}$$

(10)

(Farquhar and von Caemmerer, 1982).

The insert in Fig. 1 illustrates the difference between $\Gamma$ and $\Gamma_{c}$. $\Gamma$ has been shown to increase with leaf age and this can be attributed to increases in the ratio $R_{d}/V_{cmax}$ and provides evidence for the occurrence of mitochondrial respiration not associated with photorespiration in vivo (Azcon-Bieto et al., 1981; Peisker, 1981).

$\Gamma_{c}$ and $R_{d}$ have been estimated from measurements of CO$_{2}$ response curves. At low intercellular CO$_{2}$ partial pressures and different irradiances the curves intersect at $\Gamma_{c}$ (Laisk, 1977). Brooks and Farquhar (1985) used this technique to determine the temperature dependence of $\Gamma_{c}$ in spinach and found that it agreed closely with the temperature dependence of $S_{o_{i}}$ of spinach made in vitro (Jordan et al., 1984$^{1}$). Laisk and Loreto (1996) made in vivo estimates of $S_{o_{i}}$ from the slope of the dependence of $\Gamma_{c}$ on oxygen concentration, but the slope is also dependent on the ratio $R_{d}/V_{cmax}$ (Eq (10)) and gives only approximate values.

Estimates of $\Gamma_{c}$ vary between 33 and 46.6 $\mu$bar at 25 °C (Evans and Loreto this book) which converts to $S_{o_{i}}$ in the range of 3181-2253 bar bar$^{-1}$, or 120–85 MM$^{-1}$. In vivo estimates of $S_{o_{i}}$ are consistently 10–20% higher than in vitro measurements and von Caemmerer et al. (1994) discuss possible explanations. Perhaps surprisingly, $\Gamma_{c}$ turns out to be a very important parameter in many aspects of mathematical modeling of CO$_{2}$ fixation even at the global scale and for that purpose more extensive measurements of temperature dependencies are required, particularly in the lower temperature range.

C. Kinetic Constants of Rubisco

1. In Vitro Estimates

The successful application of Eq. (7) requires accurate knowledge of the Michaelis Menten constants for

---

1 Because Rubisco kinetics relate to the CO$_{2}$ partial pressure at the site of carboxylation in the chloroplast, which at $\Gamma_{c}$ is slightly greater than the measured intercellular CO$_{2}$ partial pressure $P_{c}$, $\Gamma_{c} = P_{c} + R_{d}/g_{i}$, where $g_{i}$ is the conductance to internal diffusion of CO$_{2}$ from the intercellular airspace to the chloroplast (von Caemmerer et al., 1994; Chapter 14, Evans and Loreto).
In vivo kinetic constants of Rubisco from several C₃ species

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_c$ (µM)</th>
<th>$K_o$ (µbar)</th>
<th>$K_a$ (µM)</th>
<th>$K_o(1+O/K_o)$ (µbar)</th>
<th>$V_{o_max}/V_{o_max}$ at 200 mbar O₂</th>
<th>$S_{co}$ (c)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atriplex glabrinsula</td>
<td>21</td>
<td>629</td>
<td>328</td>
<td>260</td>
<td>1113</td>
<td>0.18</td>
<td>85 Badger and Collatz, 1977</td>
</tr>
<tr>
<td>Spinacea oleracea</td>
<td>13.6</td>
<td>407</td>
<td>354</td>
<td>280 (a)</td>
<td>698</td>
<td>0.22</td>
<td>120 Badger and Andrews, 1974</td>
</tr>
<tr>
<td>Glycine max</td>
<td>9</td>
<td>269</td>
<td>430</td>
<td>341 (a)</td>
<td>427</td>
<td>0.58</td>
<td>82 Jordan and Ogren, 1981</td>
</tr>
<tr>
<td>Tetragnonium expansa</td>
<td>13</td>
<td>389</td>
<td>600</td>
<td>476 (a)</td>
<td>552</td>
<td>0.55</td>
<td>81</td>
</tr>
<tr>
<td>S. oleracea</td>
<td>14</td>
<td>419</td>
<td>480</td>
<td>381 (a)</td>
<td>639</td>
<td>0.43</td>
<td>80</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>16</td>
<td>479</td>
<td>500</td>
<td>397 (a)</td>
<td>720</td>
<td>0.38</td>
<td>80</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>11</td>
<td>329</td>
<td>650</td>
<td>516 (a)</td>
<td>456</td>
<td>0.77</td>
<td>77</td>
</tr>
<tr>
<td>S. oleracea</td>
<td>11</td>
<td>329</td>
<td>500</td>
<td>397 (a)</td>
<td>495</td>
<td>0.52</td>
<td>88 Jordan and Ogren, 1984</td>
</tr>
<tr>
<td>Triticum aestivum</td>
<td>11.2</td>
<td>335</td>
<td>383</td>
<td>304</td>
<td>555</td>
<td>0.29</td>
<td>120 Makino et al., 1988</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>8.0</td>
<td>239</td>
<td>335</td>
<td>266</td>
<td>418</td>
<td>0.33</td>
<td>128</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>11.5</td>
<td>344</td>
<td>222</td>
<td>176</td>
<td>713</td>
<td>0.24</td>
<td>81 Whitney et al., 1998</td>
</tr>
</tbody>
</table>

All values were measured at 25 °C. To convert $K_c$ and $K_o$ values from concentration to partial pressures the solubilities for CO₂ of 0.0334 mol (1 bar)⁻¹ and for O₂ of 0.00126 mol (1 bar)⁻¹ were used. $K_c$ values by Badger and Collatz (1977) and Badger and Andrews (1974) where measured in 100 mM Hepes pH 8.3 and were recalculated here with a pKa = 6.12. Measurements by Jordan and Ogren (1981, 1984) were made with 50 mM Bicine buffer pH 8.3 and a pH = 6.23. Measurements by Makino et al. (1988) were made in 100 mM Bicine pH 8.15 and a pH = 6.12 was used.

Phosphorylation and dephosphorylation of Rubisco are carried out by the chloroplast stroma at high concentrations, which are difficult to mimic in vitro. Therefore, it is attractive to study Rubisco in its natural environment. Previously this has not been possible because other processes, such as the regeneration of RuBP, at high CO₂ partial pressures (Fig. 2) limit CO₂-assimilation rate and carboxylation. However in transgenic tobacco with reduced amounts of Rubisco the capacity for RuBP regeneration is high relative to Rubisco activity such

<table>
<thead>
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<th>Species</th>
<th>$K_c$ (µM)</th>
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</tr>
<tr>
<td>Tetragnonium expansa</td>
<td>13</td>
<td>389</td>
<td>600</td>
<td>476 (a)</td>
<td>552</td>
<td>0.55</td>
<td>81</td>
</tr>
<tr>
<td>S. oleracea</td>
<td>14</td>
<td>419</td>
<td>480</td>
<td>381 (a)</td>
<td>639</td>
<td>0.43</td>
<td>80</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>16</td>
<td>479</td>
<td>500</td>
<td>397 (a)</td>
<td>720</td>
<td>0.38</td>
<td>80</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>11</td>
<td>329</td>
<td>650</td>
<td>516 (a)</td>
<td>456</td>
<td>0.77</td>
<td>77</td>
</tr>
<tr>
<td>S. oleracea</td>
<td>11</td>
<td>329</td>
<td>500</td>
<td>397 (a)</td>
<td>495</td>
<td>0.52</td>
<td>88 Jordan and Ogren, 1984</td>
</tr>
<tr>
<td>Triticum aestivum</td>
<td>11.2</td>
<td>335</td>
<td>383</td>
<td>304</td>
<td>555</td>
<td>0.29</td>
<td>120 Makino et al., 1988</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>8.0</td>
<td>239</td>
<td>335</td>
<td>266</td>
<td>418</td>
<td>0.33</td>
<td>128</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>11.5</td>
<td>344</td>
<td>222</td>
<td>176</td>
<td>713</td>
<td>0.24</td>
<td>81 Whitney et al., 1998</td>
</tr>
</tbody>
</table>

In vitro measurements of $K_o$ and $K_a$ vary widely. Yeoh et al. (1981) measured $K_o$ in 28 different C₃ species and reported values from 359 to 778 µbar (12-26 µM). Jordan and Ogren (1981) measured a range from 269 to 479 µbar (9-16 µM) for five C₃ species. Measurements by Seemann et al. (1981) and Seemann and Berry (1982) were 310 µbar (10.4 µM). Makino et al. (1988) measured $K_c$ by a rapid assay with crude leaf extracts in several C₃ species and found values from 203 to 326 µbar (6.8–10.9 µM).

In Table 1, in vitro kinetic constants of Rubisco are collated from experiments where both carboxylase and oxygenase activity have been measured. The oxygenase activity of Rubisco has not been measured as frequently as carboxylation and fewer in vitro values of $K_o$ exist in the literature. The measured values of $K_o$ range from 156 mbar (196 µM) to 516 mbar (650 µM). The $K_o$ of Rubisco is an important parameter determining the CO₂-assimilation rate. In conjunction with $K_c$, it defines the apparent Michaelis Menten constant for CO₂, $K_c(1 + O/K_o)$ of Rubisco in the presence of O₂. In vitro measurements of $K_o(1 + O/K_o)$ are about twice the intercellular CO₂ concentration usually observed in leaves at high light (Table 1).

The greatest uncertainty in the kinetic constants surrounds the ratio of $V_{o_max}/V_{o_max}$. Jordan and Ogren (1981, 1984) calculated this value from their measurements of $S_{co}$, $K_c$ and $K_o$ and arrived at values of 0.54. Lower values of 0.18 to 0.33 have been estimated by Badger and coworkers, Makino et al. (1988) and Whitney et al. (1998) (Table 1).

2. In Vivo Estimates

The study of Rubisco by in vitro methods encounters problems since extraction and purification may cause loss of activity. Furthermore, Rubisco exists in the chloroplast stroma at high concentrations, which are difficult to mimic in vitro. It is therefore attractive to study Rubisco in its natural environment. Previously this has not been possible because other processes, such as the regeneration of RuBP, at high CO₂ partial pressures (Fig. 2) limit CO₂-assimilation rate and carboxylation. However in transgenic tobacco with reduced amounts of Rubisco the capacity for RuBP regeneration is high relative to Rubisco activity such
that CO₂ assimilation rate is Rubisco limited at all measured CO₂ partial pressures (Fig. 2, Hudson et al., 1992). Measurements of CO₂ response curves at different O₂ concentrations in these plants mimic the expected kinetic behavior of Rubisco in vitro (Figs. 3 and 4). The in vivo estimates of $K_c$ and $K_s$ fall within the range of in vitro measurements but are at the low end (Table 2). The estimate of $V_{\text{max}}/V_{\text{cmax}}$ from $\Gamma^*$, $K_c$ and $K_s$ agree best with the low in vitro estimates of Badger and coworkers (Table 1).

It is possible to quantify Rubisco catalytic site content per unit leaf area with the aid of $^{14}$C labeled carboxy arabinitol-1,5-bisphosphate (CABP). CABP is a reaction intermediate analogue that binds tightly and stoichiometrically to Rubisco catalytic sites. Furthermore since CABP binds significantly more tightly to carbamylated Rubisco sites it is possible to exchange [14C] CABP from non carbamylated sites with an excess of unlabelled CABP and quantify the number of carbamylated sites as well (Collatz et al., 1979; Butz and Sharkey, 1989). The maximal Rubisco activity, $V_{\text{cmax}}$, is the product of the Rubisco site content multiplied by the catalytic turnover rate $k_{\text{cat}}$ (mol CO₂/(mol Rubisco sites)). In vitro maximal Rubisco activity can be measured in leaf extracts from the rate...
of $^{14}$CO₂ incorporation into acid stable products after pre-incubation of the extract with CO₂ and magnesium to achieve full carbamylation. Combined measurements of Rubisco activity and Rubisco site content after rapid extraction of Rubisco have given in vitro $k_{cat}$ values between 2 and 6 mol CO₂ (mol sites)$^{-1}$s$^{-1}$ (Table 2, Evans and Seemann, 1984; Makino et al., 1988; Whitney et al., 1999). Transgenic tobacco plants with reduced amounts of Rubisco have provided an opportunity to measure the $k_{cat}$ of Rubisco carboxylase in vivo from combined measurements of CO₂ assimilation rates at high CO₂ partial pressure and measurements of Rubisco site concentrations. The values by von Caemmerer et al. (1994) given in Table 2 are higher than most in vitro values but less than those measured by Whitney et al. (1998).

### D. Dependence of Carboxylation Rate on RuBP Concentration

Rubisco has a high affinity for RuBP with an in vitro Michaelis Menten constant, $K_c$ ~ 20 μM (Badger and Collatz, 1977; Yeoh et al., 1981). However many PCR and PCO cycle intermediates such as FBP, NADPH, 6-phosphogluconate, Pi, and PGA bind to Rubisco’s catalytic sites and act as competitive inhibitors which increases the apparent $K_c$ (Badger and Lorimer, 1981). Chloroplasts contain large pools of RuBP and PGA which can both be around 10 mM. This PGA concentrations together with a $K_p$ of 0.9 mM is sufficient for a ten-fold increase in $K_c$ (Figs. 5, 6 and 7; Badger et al., 1984; von Caemmerer and Edmondson, 1986; Seemann and Sharkey, 1986; Price et al., 1995). The Rubisco site concentrations in vivo are between 1–4 mM (Evans et al., 1994) and at these concentrations a significant amount of RuBP is bound to Rubisco sites and simple Michaelis Menten kinetics do not apply. Farquhar (1979) showed that because of the low $K_p$, the kinetics of Rubisco with respect to free and Rubisco bound RuBP should be such that the carboxylation rate increases linearly with free and bound RuBP until the RuBP pool reaches Rubisco site concentration and then saturates abruptly when the RuBP concentrations exceed the

---

**Table 2.** Rubisco kinetic constants at 25 °C calculated from gas exchange analysis of transgenic tobacco with reduced Rubisco content

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_c$</td>
<td>258 ± 50 μbar, (8.6 ± 1.7 μM)</td>
</tr>
<tr>
<td>$K_p$</td>
<td>171 μbar, (215 μM)</td>
</tr>
<tr>
<td>$K_c (1 + O/K_o)$ at 200 mbar O₂</td>
<td>560 μbar, (18.7 μM)</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>38.6 μbar</td>
</tr>
<tr>
<td>$S_{E_{00}}$</td>
<td>97.5</td>
</tr>
<tr>
<td>$V_{max}/V_{cmax}$</td>
<td>0.255</td>
</tr>
<tr>
<td>$k_{cat} (mol CO₂ (mol sites)^{-1}s^{-1})$</td>
<td>3.53 ± 0.18</td>
</tr>
<tr>
<td>$k_{cat} (mol CO₂ (mol sites)^{-1}s^{-1})$</td>
<td>2.87 ± 0.08</td>
</tr>
</tbody>
</table>

(a) To convert values from partial pressures to concentrations, solubilities for CO₂ of 0.0334 mol (l bar)$^{-1}$ and for O₂ of 0.00126 mol (l bar)$^{-1}$ were used (adapted from von Caemmerer et al., 1994).
site concentration (Fig. 5). This dependence on total chloroplastic RuBP makes it possible to describe CO₂ assimilation rate by either the RuBP-saturated or the RuBP-regeneration limited rate of Rubisco (Eqs. (7) and (9)). It has been difficult to test this hypothesis in vivo. When varying irradiance varied RuBP regeneration rates, Rubisco carboxylation also changed (Mott et al., 1984; von Caemmerer and Edmondson, 1986). The first confirmation, that the dependence of carboxylation rate on chloroplastic RuBP concentration was similar to that predicted by the model equation shown in Fig. 5 came from an elegant study by Mott et al. (1984). They examined

the rapid adjustment in CO₂ assimilation rate and RuBP pools after a light transient at time points before a change in Rubisco carboxylation had occurred. Transgenic tobacco plants with an antisense reduction in chloroplastic glyceraldehyde-3-phosphate dehydrogenase activity (anti-GAPDH plants) have reduced rates of RuBP regeneration and RuBP pools without concomitant changes in Rubisco carboxylation. The relationship between assimilation rate and RuBP pools generated from wild type and anti-GAPDH plants is also consistent with the hypothesis of Farquhar (1979) especially when the $K_r$ is increased to take into account the competitive inhibition by PGA (Fig. 5).

There has been some debate over how much RuBP is required to saturate Rubisco sites. Several studies have suggested that RuBP needs to be more than 1.5–2 times the Rubisco site concentration (Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986; Seemann et al., 1987; Price et al., 1995) which is consistent with the data in Fig. 5. The RuBP and PGA pools vary drastically and in opposite direction with CO₂ concentration, with little change in the total esterified phosphate pool. This is probably governed by the strict exchange of triose phosphate and phosphate by the phosphate translocator at the chloroplast envelope (Chapter 6, Flügge). In vivo the RuBP pool is very large at low CO₂ partial pressures and low at high CO₂ partial pressures, and under these conditions PGA pools are also very high (Fig. 6; Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986; Seemann et al., 1987). RuBP and PGA change much less with changing irradiance since several of the PCR cycle enzymes are light regulated (Fig. 7, Stitt, 1996). Curiously, in all species examined, RuBP pool size rarely drops below Rubisco site concentration, which may have something to do with Rubisco’s requirement for RuBP to maintain full carboxylation (see below).

### E. CO₂ Assimilation Rate Versus Rubisco Activity

In early gas-exchange studies, it was assumed that CO₂ assimilation rate at high light was limited by the physical constraints of CO₂ diffusion (Gaastra 1959). However, it quickly became apparent that there was also a close correlation between CO₂ assimilation rates and maximal extractable Rubisco activity (reviewed by Björkman, 1981). Fig. 8a and b show such correlations for *Phaseolus vulgaris* and
Nicotiana tabaccum, but there are many other examples in the literature (Seemann et al., 1981; Makino et al., 1985, 1994b; Evans and Terashima, 1988). Usually measurements are made at a constant ambient CO₂ partial pressure and the relationship tends to be somewhat curvilinear as the chloroplast CO₂ partial pressure may be less in leaves with greater amounts of Rubisco (Evans et al., 1986; Evans and Terashima, 1988; Makino et al., 1994b). A linear relationship is only expected when measurements are made at a constant chloroplastic CO₂ partial pressure (Eq. (7)). However, when low light measurements of CO₂ assimilation rate are correlated with maximal Rubisco activity, the relationship saturates abruptly as the rate of RuBP regeneration and not Rubisco limits the rate of CO₂ assimilation (Fig. 1; Laurer et al., 1993). It is important to note that the maximal Rubisco activity needs to be at least four times the rate of CO₂ assimilation at ambient CO₂ and 25 °C to account for subsaturating CO₂ concentrations (Eq. (7), Table 1). In many species it can be difficult to extract sufficient functional Rubisco to account for CO₂ assimilation rates in leaves.

The close correlation between Rubisco and CO₂ assimilation rate occurs because there is little variation in the chloroplastic CO₂ partial pressure at high irradiance (Chapter 14, Evans and Loreto). However some exceptions can be found. For example the obligate CAM plant Kalanchoe diagremontiana, which can operate in the mode during phase IV of the CAM cycle, has a very low internal conductance to diffusion. Although Kalanchoe leaves have about the same amount of Rubisco as tobacco leaves, CO₂ assimilation rate is only half that of a tobacco leaf at ambient CO₂ partial pressure (Fig. 9, Maxwell et al., 1997). Makino et al. (1994a) noted that when rice was grown at different temperatures the relationship between CO₂ assimilation rate and
extractable Rubisco activity changed and attributed the difference to a possible increase in internal resistance to CO₂ diffusion at low temperatures.

F. Temperature Dependence

The temperature dependencies of Rubisco’s carboxylation and oxygenation rates are reflected in the temperature dependency of the CO₂ assimilation rate of leaves (Björkman and Pearcy, 1971; Björkman et al., 1980). The need for accurate estimates of the temperature dependencies of Rubisco kinetic parameters has become apparent as mathematical modelers try to predict the impact of increasing global CO₂ concentrations and temperatures (Bowes, 1991; Long, 1991; Walcroft et al., 1997). The temperature dependence of the kinetic constants can be described by an Ahrrenius function of the form given below (Badger and Collatz, 1977).

\[
\text{Parameter} = \frac{\text{Parameter (25 °C)}}{\exp[(T - 298)E/(298RT)]},
\]

where \( R \) (8.314 J K⁻¹ mol⁻¹) is the universal gas constant and \( T \) is temperature in Kelvin (K). Table 3 shows the activation energies (\( E \)) of Rubisco kinetic constants determined in in vitro experiments. There are only two complete data sets measured on C₃ plants, that of Badger and Collatz (1977) on Rubisco from Atriplex and that by Jordan and Ogren (1974) on spinach Rubisco.

The specificity factor of Rubisco decreases with increasing temperature (Eq. (2)). This is because the reaction mechanism of Rubisco involves a 2,3 enediol intermediate and its reaction with O₂ has a higher free energy of activation than the reaction with CO₂ (Andrews and Lorimer, 1987). That is, the oxygenation rate increases more rapidly with temperature than the rate of carboxylation. Fig. 10 shows the
temperature dependence of Rubisco limited CO₂ assimilation rate (Eq. (7)) and Γ, using the various activation energies given in Table 3. Predictions vary widely at the higher temperatures. However, it is likely that CO₂ assimilation rate is limited by the RubBP regeneration capacity at high temperature, in which case the temperature dependency of Γ is important. Again predictions vary widely at the higher temperatures. The temperature dependency of Γ, determined by gas exchange measurements by Brooks and Farquhar (1985) agree moderately well with the estimates of Γ from the measurements of Rubisco specificity by Jordan and Ogren (1984).

Comparing the activation energies in Table 3 shows that the ratio of \( V_{\text{max}} / K_c \) does change much with temperature. This is supported by gas exchange measurements that show the initial slope of the CO₂ response curve of CO₂ assimilation to be relatively insensitive to temperature (von Caemmerer and Farquhar, 1981; Kirschbaum and Farquhar, 1984; Sage et al., 1990a). It is perhaps interesting to note that the lower value of \( S_{\text{eto}} \) of the photosynthetic
To function, Rubisco’s catalytic sites must be activated through the reversible carbamylation of a lysine residue within the site, followed by the rapid binding of an essential $\text{Mg}^{2+}$ (Andrews and Lorimer, 1987). It is possible to measure both Rubisco activity and the carbamylation state of Rubisco in leaf extracts (Butz and Sharkey, 1989). Rubisco is usually fully active and carbamylated at ambient $\text{CO}_2$ concentrations and high light (Figs. 5 and 6). By itself, Rubisco would not be fully carbamylated in vivo. The analysis

### III. In Vivo Regulation of Rubisco Carbamylation and Activity

#### A. Regulation of Rubisco Activation State by RuBP and Activase

To function, Rubisco’s catalytic sites must be activated through the reversible carbamylation of a lysine residue within the site, followed by the rapid binding of an essential $\text{Mg}^{2+}$ (Andrews and Lorimer, 1987). It is possible to measure both Rubisco activity and the carbamylation state of Rubisco in leaf extracts (Butz and Sharkey, 1989). Rubisco is usually fully active and carbamylated at ambient $\text{CO}_2$ concentrations and high light (Figs. 5 and 6). By itself, Rubisco would not be fully carbamylated in vivo. The analysis

### Table 3. Activation energies $E$ (J/mol) of Rubisco kinetic constants

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_c$</th>
<th>$K_s$</th>
<th>$V_{\text{max}}$</th>
<th>$V_{\text{max}}$</th>
<th>$S_{\text{ci}}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine max (15–35 °C)</td>
<td>53,381</td>
<td>8,279</td>
<td>54,393</td>
<td>79,497</td>
<td>Laing et al., 1974</td>
<td></td>
</tr>
<tr>
<td>Spinacia oleracea (5–35 °C)</td>
<td>59,414</td>
<td>35,983</td>
<td>64,853</td>
<td>59,414</td>
<td>Badger and Andrews, 1974</td>
<td></td>
</tr>
<tr>
<td>Atriplex glabrifolia (above 15 °C)</td>
<td>10,962</td>
<td>35,983</td>
<td>103,765</td>
<td>103,347</td>
<td>Badger and Collatz, 1977</td>
<td></td>
</tr>
<tr>
<td>(below 15 °C)</td>
<td>81,655</td>
<td>15,632</td>
<td>74,350</td>
<td>37,566</td>
<td>Jordan and Ogren, 1984</td>
<td></td>
</tr>
<tr>
<td>Spinacia oleracea (7–35 °C)</td>
<td>59,356</td>
<td>35,948</td>
<td>58,520</td>
<td>58,520</td>
<td>Farquhar et al., 1980</td>
<td></td>
</tr>
<tr>
<td>model plant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values of parameters can be calculated at any temperature from the following equation:

$$\text{Parameter} = \text{Parameter} (25 \degree \text{C}) \exp[(T - 298)/E]/\exp(298/R),$$

where $R$ \((8.314 \text{ J K}^{-1} \text{ mol}^{-1})\) is the universal gas constant and $T$ is temperature in degrees Kelvin (K). $Q_{10}(25 \degree \text{C}) = \exp(13.6 \times 10^{-6} E)$

bacteria _Rhodospirillum rubrum_ is accompanied by a lower activation energy (Chen and Spreitzer, 1992; Lorimer et al., 1993). There have so far been no studies that have looked for species-specific differences in Rubisco’s temperature dependency amongst higher plants. Small differences of $S_{\text{ci}}$ at 25 °C could yield more important differences at higher temperatures. The temperature dependence of $\Gamma$, increases the $\text{CO}_2$ compensation point at high temperatures making it difficult for plants to maintain positive $\text{CO}_2$ assimilation rate under conditions where conservation of water is paramount and closure of stomata would be beneficial. The temperature dependence of $\Gamma$, is also reflected in the strong temperature dependency of the quantum yield of $C_3$ plants (Ehleringer and Björkman, 1977; Farquhar et al., 1980 and Oberhuber and Edwards, 1993) where more electron transport has to be devoted to PCO cycle activity as temperatures increase.

#### Fig. 10. Modeled rates of Rubisco limited $\text{CO}_2$ assimilation and $\Gamma$, with increasing temperature. $\text{CO}_2$ assimilation rate (Eq. (7) with $R_d = 0$) was calculated with the activation energies given in Table 3 and $V_{\text{max}} = 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ at two different chloroplastic $\text{CO}_2$ partial pressures. The following kinetic constants were used: Badger and Collatz (1977) and Farquhar et al. (1980) $K_c = 460 \mu\text{bar}$, $K_s = 330$ and $\Gamma* = 31 \mu\text{bar}$, Jordan and Ogren (1984); $K_c = 419 \mu\text{bar}$, $K_s = 381 \mu\text{bar}$ and $\Gamma* = 47 \mu\text{bar}$, Brooks and Farquhar 1985: a $\Gamma* = 42.7 + 1.68(T - 25) + 0.012(T - 25)^2$, b $44.7 + 1.88(T - 25) + 0.036(T - 25)^2$. 

of the in vitro kinetics of Rubisco has shown that the equilibrium carbamylation in the absence of RuBP would be less than 25% at the pH, Mg$^{2+}$, and CO$_2$ concentrations thought to occur in the chloroplast (Lorimer et al., 1976). Besides, when RuBP was present in in vitro assays, it appeared to block carbamylation by binding tightly to non-carbamylated sites (Jordan and Chollet, 1983).

These in vitro difficulties in Rubisco carbamylation are eliminated in vivo by the presence of a second protein, Rubisco activase (Portis, 1990, Salvucci and Ogren, 1996). Activase was first identified in a high CO$_2$ requiring mutant of Arabidopsis thalina (Sommerville et al., 1982) which had poorly activated Rubisco in the light (Salvucci et al., 1985). Studies with the purified protein have demonstrated that activase requires ATP hydrolysis to function (Streusand and Portis, 1987) and that activase specifically removes sugar phosphates from carbamylated and uncarbamylated Rubisco sites, thereby promoting carbamylation (Portis et al., 1986; Portis, 1990).

1. Activase Mode of Action

Andrews et al. (1995), Mate et al. (1996) and Salvucci and Ogren (1996) proposed models for the mechanism of activase action. They suggested a mechanistic model in which activase functions to open closed polypeptide loops within the protein structure at the catalytic site that normally enclose tight binding ligands. It is hypothesized that when activase is activated by ATP hydrolysis, it can recognize Rubisco with a closed loop protein structure and bind selectively to these catalytic sites. This causes the loops to open, releasing the ligand and returning activase to its inactive form (Fig. 11). The closed loop complexes that activase recognizes can occur with both carbamylated (ECM) and uncarbamylated (E) Rubisco and the unassisted rate at which they release their ligands is very slow (see legend of Fig. 12 for definition of terms). When the ligand is the substrate, RuBP, catalysis provides a rapid means of opening loops for the ECMR complex and which does not require the assistance of activase (Fig. 12). Therefore, activase will induce a much larger increase in the rate of opening of the unproductive ER complexes than it will in the rate of opening of the catalytically-competent, carbamylated complexes. This difference provides the opportunity for Rubisco’s carbamylation status to respond to the concentration of RuBP as well as the activity of activase. Mate et al. (1996) formulated this mechanistic model in simple mathematical terms.
using the sequence of carbamylation and catalytic reactions originally proposed by Farquhar (1979). The model suggests that at ambient CO₂ concentration and high RuBP concentrations, carbamylation levels approximating those observed in vivo can only occur when the apparent dissociation constant of the ER complex (K₀) is high relative to Kᵣ (Fig. 13). Since, as discussed above, an activase-mediated increase in the rate of ligand dissociation of the ER complex is likely to be larger than the dissociation of the ECMR complex, where catalysis provides an alternative route, activase activity can lead to an increase in the apparent ratio Kᵣ/K₀.

2. The Role of RuBP in Promoting Carbamylation

The suggestion is frequently made that Rubisco carbamylation is modulated to match the rate of RuBP consumption to that of RuBP regeneration (Sage, 1990). The model by Mate et al. (1996) also proposes a role for RuBP concentration in the regulation of carbamylation state (Fig. 13). When activase is active (i.e. K₀/Kᵣ is large) Rubisco’s carbamylation status becomes strongly dependent on RuBP concentration when RuBP concentrations fall below the enzyme site concentration. This may provide a very responsive mechanism for ensuring that Rubisco’s activity is modulated according to the prevailing rate of RuBP supply, which may vary with environmental conditions, particularly irradiance. For example Mott et al. (1984) showed that a light transient from high to low light resulted in an immediate drop in RuBP concentration followed by deactivation of Rubisco. Deactivation of Rubisco following a transient drop in RuBP pools were also observed by Sharkey et al. (1986) when leaves were transferred to low O₂ concentrations. Portis et al. (1995) reported that Rubisco decarbamylated in vitro at subsaturating concentrations of RuBP.

If the ratio K₀/Kᵣ is less than unity, the extent of carbamylation is predicted to be less in the presence of RuBP than that observed in the absence of RuBP (Fig. 13). This highlights the importance of activase, since in vitro measurements of Kᵣ (20 nM, Jordan and Chollet, 1983) show it to be two orders of magnitude smaller than Kᵣ.

3. How Much Activase is Required to Keep Rubisco Carbamylated

Tobacco leaves have between 40–100 mg m⁻² of activase, which constitutes approximately 1–2% of leaf soluble protein. If it is assumed that activase is a tetramer with subunits size of 47 kD (Portis, 1990) then the stoichiometry of activase to Rubisco is one activase tetramer to 80 Rubisco catalytic sites. In Arabidopsis activase constitutes about 5% of soluble protein and leaves have between 100 to 200 mg m⁻², which gives a ratio of one to 20-30 (Eckhard et al., 1997). Activase could be reduced to below 5% of wildtype levels in transgenic tobacco before effects on the steady state levels of Rubisco carbamylation and CO₂ assimilation rate could be seen (Mate et al., 1996, Fig. 14) and similar results were observed in Arabidopsis (Eckhard et al., 1997). Thus activase must be acting in the manner of a catalyst, since there is insufficient activase for it to be a permanently bound ligand. Much higher ratios of activase to Rubisco are required in vitro to promote carbamylation (Portis et al., 1986; Lan and Mott, 1991).

Although only low levels of activase are required to achieve full carbamylation in the steady state, studies have shown that even small reductions in activase lead to a decrease in the rate of Rubisco activation (Hammond et al., 1995; Jiang et al., 1996; Hammond et al., 1998). Furthermore Andrews et al. (1995) and He et al. (1997) have shown that leaves of transgenic tobacco plants with very low amounts of
activase have rates of CO₂ assimilation very much lower than that predicted from the carbamylated Rubisco site content. They were not able to resolve whether this was due to an inhibitor bound to carbamylated sites which was destroyed upon extraction and it leaves the question open to whether activase has another as yet unknown role in Rubisco catalysis in vivo.

B. Rubisco Activation State at Different Environmental Conditions

1. Variation in Rubisco Activation State with Irradiance

The activation state (measured by comparison of initial and in vitro activated Rubisco activity) and the carbamylation state of Rubisco (measured by CABP binding as described in Section II.C.2) are low in the dark and increase with irradiance (Perchorowitz et al., 1981; Mott et al., 1984; von Caemmerer and Edmondson, 1986; Seemann, 1989; Sassenrath-Cole et al., 1994, Krall et al., 1995). Rubisco is never completely decarbamylated in the dark and the carbamylation state is between 30 and 40% depending upon species. The carbamylation level of Rubisco rarely limits steady state rates of CO₂ assimilation and under conditions where RuBP regeneration capacity is low a decrease in carbamylation occurs (Mott et al., 1984; Sage, 1990; Sassenrath-Cole et al., 1994). Changes in stromal pH and magnesium, which saturate at very low irradiances, are insufficient to account for observed changes in Rubisco carbamylation and activity (Woodrow and Berry, 1988). It is likely that both changes in RuBP pool size (and other metabolites such as PGA) and activase activity are involved in the modulation of carbamylation and activity as outlined in section III (Portis, 1992; Salvucci and Ogren, 1996).

At low CO₂ concentrations Rubisco can be almost fully carbamylated at low light (Sage et al., 1990b). This is also evident in gas exchange measurements which show that the initial slope of the CO₂ response curve is frequently independent of irradiance down to quite low irradiance levels (Brooks and Farquhar, 1985; Evans, 1986; Sage et al., 1990b). Under these conditions the capacity for RuBP regeneration exceeds the carboxylation capacity and presumably the transthylakoid ΔpH as well as the ATP/ADP ratio are likely to be high which in turn may translate into a greater activase activity. In the same vein, the carbamylation state was found to be high at low irradiance in transgenic tobacco plants with reduced amount of Rubisco (Quick et al., 1991a) whereas there was no difference at high light (von Caemmerer et al., 1992). For shade leaves the irradiance required for full activation of Rubisco appears to be lower than in sun leaves (Seemann, 1989; Krall, 1995), although it is unclear what mechanism underpins this adaptation.

Leaves of some species contain substantial amounts of a nocturnal inhibitor carboxyarabinitol 1-phosphate (CA1P) (Vu et al., 1984; Gutteridge et al., 1986; Servaites et al., 1986; Berry et al., 1987). CA1P binds tightly to the carbamylated (ECM) form of Rubisco and reduces the Rubisco activity that remains after extraction and subsequent in vitro activation by CO₂ and magnesium (Seemann and Berry, 1982; Berry et al., 1987; Seemann et al., 1990; Servaites, 1990). Leaves of soybean, Phaseolus, tobacco and
Alocasia contain large amounts of CA1P, while other species (e.g. spinach, wheat and Arabidopsis) contain very little (Vu et al., 1984; Servaites et al., 1986). The easiest assay for the presence of the nocturnal inhibitor is the combined measurement of Rubisco site concentration and in vitro activated Rubisco activity. A reduction of the catalytic turnover rate is a good indicator for the presence of a CA1P (Seemann, 1989; Seemann et al., 1990). In soybean, CA1P has been shown to form part of the diurnal light regulation of Rubisco (Servaites et al., 1991). High irradiance leads to the rapid removal of CA1P from Rubisco sites. However, in the natural daylight regime there is a more gradual decline in CA1P during the morning which contributes to the observed gradual rise in Rubisco activity (Kobza and Seemann, 1989; Servaites et al., 1991; Sage et al., 1993). Activase has been shown to release CA1P from the ECM complex in vitro (Robinson and Portis, 1988). In transgenic tobacco plants with reduced amounts of activase, CA1P was removed from Rubisco sites more slowly following illumination than in control plants (Mate et al., 1993).

After a period of illumination CA1P is low and requires several hours to increase again when irradiance is decreased and thus CA1P does not seem to play a role in short term modulation of Rubisco activity or of Rubisco regulation at dusk (Sage et al., 1993). Servaites (1990) and Moore and Seemann (1992) have reviewed what is known about the metabolism of CA1P. The role that CA1P plays in the regulation of Rubisco activity remains unclear. It is obviously not essential for the light regulation of Rubisco in many species.

2. Variation of Rubisco Activation State with CO2

In vitro, Rubisco carbamylation is strongly dependent on the prevailing CO2 and magnesium concentration (Laing and Christeller, 1976; Lorimer et al., 1976). The same dependence on CO2 concentration is however not observed in vivo where RuBP and activase activity are thought to maintain Rubisco activation state (Section III.A; Portis, 1992). However reduced Rubisco activation state has been observed at CO2 concentrations below the CO2 compensation point (Fig. 6; von Caemmerer and Edmondson, 1986). A decrease in activation state can also be observed at high CO2 concentrations (Fig. 6; von Caemmerer and Edmondson, 1986; Sage et al., 1988, 1990).

Sage et al. (1990) noted that the deactivation at high CO2 was more pronounced at low light and suggested that Rubisco decarbamylation occurred to match the rate of Rubisco with the rate of RuBP regeneration to regulate metabolite concentrations. This provides a plausible explanation for the phenomenon but the mechanism of how this is achieved remains unclear. In studies with transgenic tobacco where RuBP regeneration rate has been reduced through a reduction in GAPDH activity, Rubisco shows no concomitant decrease in Rubisco carbamylation (Price et al., 1995). However in this case the ATP/ADP ratios are high and perhaps this is a further indication that Rubisco carbamylation and activity is closely tied to activase activity which in turn is influenced by the ATP/ADP ratio and perhaps the redox state of the electron transport chain (Portis, 1992).

Sharkey et al. (1986a) showed that the extent of Rubisco carbamylation also decreased when CO2 assimilation rate was limited by the export rate of triose phosphate out of the chloroplast. Under these conditions, which can be induced by a combination of low O2 concentration and elevated CO2 concentration, free stromal phosphate and ATP are in short supply (Leegood and Furbank, 1986; Sharkey et al., 1986b; Sharkey and Vanderveer, 1989). Sawada et al. (1990) suggested that the deactivation of Rubisco seen in sink-limited soybean plantlets may also be caused by reduced levels of free phosphate.

3. Time Course of Activation

In vivo, Rubisco activation and deactivation is slow with time constants between four and five min for the activation process and between 20 and 25 min for deactivation process. The light activation and deactivation of other PCR cycle enzymes occurs much more rapidly (for review see, Pearcy et al., 1996). During photosynthetic light induction experiments good correlations have been found between CO2 assimilation rate and initial Rubisco activity (the maximal Rubisco activity measured in leaf extracts after rapid extraction and without in vitro activation by CO2 and magnesium)(Pearcy, 1988; Woodrow and Mott, 1989). Both Rubisco’s slow activation and the even slower deactivation have a profound effect on the CO2 fixation capacity of leaves in fluctuating light environments and Pearcy and coworkers have provided detailed analyses (Pearcy, 1990; Sassenrath-Cole et al., 1994; Krall et
al., 1995; Pearcy et al., 1996). No consistent differences in the rate of Rubisco activation and deactivation have been observed between species from different habitats, and not even between species with and without CA1P metabolism (Kobza and Seemann, 1989; Pearcy et al., 1996; Ernsten et al., 1997). This is surprising since studies with transgenic tobacco and Ambidopsis plants with reduced amounts of activase content have shown that the rate of Rubisco activation is almost linearly dependent on the activase content in these plants. There thus appears to be the possibility to modulate the rate of activation. Plants with very low activase content showed very slow rates of photosynthetic induction (Hammond et al., 1995, 1998; Mott et al., 1997). These results also show that whilst activase may have minor control on photosynthetic rate in the steady-state, its importance increases dramatically in plants experiencing fluctuating rates of photosynthesis due to changing environmental conditions (e.g. below a leaf canopy) where rapid changes in irradiance frequently occur (Hammond et al., 1998).

**IV. Rubisco Content of Leaves of Plants Grown in Different Environmental Conditions**

**A. Nitrogen Nutrition**

The majority of leaf nitrogen is tied up in the photosynthetic components of the chloroplasts (Chapin et al., 1987; Evans, 1989, 1996; Evans and Seemann, 1989). In C₅ plants approximately 25% of nitrogen is invested in Rubisco alone. The CO₂ concentrating mechanism makes C₄ plants more nitrogen use efficient and Rubisco constitutes only 10 to 15% of leaf nitrogen (Sage et al., 1987). Nitrogen is frequently a scarce commodity and it is not surprising that the Rubisco content of leaves declines with leaf age as nitrogen is remobilized to young developing leaves. There are many studies that have examined the changes in CO₂ assimilation rate with leaf age (Evans, 1983; Makino et al., 1985; Hidema et al., 1991). Rubisco content of leaves increases in expanding leaves and then declines again as leaves senesce and there is usually a strong correlation between CO₂ assimilation rate and Rubisco activity or content and other photosynthetic components (Makino et al., 1985; Hidema et al., 1991; He et al., 1997).

Different nitrogen nutrition also has, among other things, a marked effect on the Rubisco content of leaves (Fig. 8; von Caemmerer and Farquhar, 1981, 1984; Björkman, 1981; Evans, 1983; Sage et al., 1987; Quick et al., 1992; Makino et al., 1994b). However, the differences in Rubisco content are also accompanied by concomitant changes in other chloroplast proteins and chlorophyll suggesting that a similar balance between the capacity for Rubisco carboxylation and RuBP regeneration is maintained. This is evident in measurements of CO₂ response curves where the initial slope and the CO₂ saturated rate change proportionally (von Caemmerer and Farquhar, 1981; Evans, 1983; Evans and Teraschima, 1988; Makino et al., 1994). The relationship between Rubisco and leaf nitrogen is usually linear with a positive intercept and differs between species (Evans, 1989). This means that leaves with a low nitrogen content invest proportionally more nitrogen into Rubisco protein. The relationship between CO₂ assimilation rate and Rubisco activity is curvilinear when measurements made at high light and constant ambient CO₂ concentration are compared (Fig. 8; Evans and Seemann, 1989; Quick et al., 1992; Fichtner et al., 1993). That is, the ratio of CO₂ assimilation rate per Rubisco content declines with increasing Rubisco activity, as other factors such as stomatal and leaf-internal conductance to CO₂ diffusion, the capacity for RuBP regeneration or the activation state of Rubisco exert more control over the rate of CO₂ assimilation (Section II.E). The mechanism by which nitrogen availability controls the abundance of Rubisco protein is largely unknown. It is interesting to note that transgenic tobacco plants with an antisense construct to Rubisco accumulate nitrate in leaves (Quick et al., 1991b; Fichtner et al., 1993; Masle et al., 1993). Recent studies have suggested a direct role of nitrate in the control of plant development and photosynthetic carbohydrate partitioning, acting at the level of gene expression (Scheible et al., 1997a,b). It remains to be identified whether such control operates on genes encoding Rubisco.

**B. Irradiance**

Plants growing under low light also have lower Rubisco content than high light grown plants (Björkman, 1981; von Caemmerer et al., 1984; Evans, 1987; Seemann, 1989; Lauerer et al., 1994; Krall et al., 1995). Chloroplasts from shade grown plants...
show a number of adaptive changes such as increased thylakoid development and increased partitioning of thylakoid protein into light capturing components away from electron transport intermediates such as cytochrome b/f complex and ATPase (Björkman, 1981; Evans and Seemann, 1989; Evans, 1996). Seemann et al. (1987) found the relationship between leaf nitrogen and Rubisco content to be independent of growth irradiance in Phaseolus and Alocasia but the shade plant Alocasia had a lower proportion of leaf nitrogen in Rubisco (10% versus 19%). However electron transport capacity, which is strongly correlated with cytochrome b/f content, is usually also correlated with Rubisco capacity and both are reduced almost in parallel under low light. This was recently confirmed for rice (Makino et al., 1997a). Although many species reduce the amount of Rubisco on a leaf area basis when transferred to, or grown at low light (von Caemmerer and Farquhar, 1984; Krall et al., 1995) most species do not reduce Rubisco sufficiently for optimal allocation of nitrogen within leaves at low light. For example, when transgenic tobacco with different antisense reductions in Rubisco was grown at 300 μmol quanta m⁻² s⁻¹, Rubisco content could be lowered well below wildtype levels before CO₂ assimilation was limited by Rubisco activity at growth light intensities (Quick et al., 1991a, Lauerer et al., 1993).

The Rubisco content of a leaf is dynamic and subject to rapid changes in response to changing environmental conditions, even within fully mature leaves. In an elegant set of experiments Prioul and Reyss (1987, 1988) demonstrated that within a single leaf of tobacco, localized changes in irradiance both up and down brought about parallel changes in the amount of Rubisco protein within a few days. These changes were paralleled by changes in the mRNA encoding the nuclear encoded small subunit of Rubisco but not the plastidic encoded large subunit mRNA. This suggested strong nuclear control of the abundance of Rubisco protein, which was highly sensitive to environmental conditions. Nuclear, rather than plastidic, control was further demonstrated when an antisense construct to the small subunit of Rubisco was shown to be sufficient to reduce the amount of Rubisco small and large subunit protein in transgenic tobacco plants, despite having no influence on the abundance of large subunit mRNA (Rodermel et al., 1988). Much work has focused on the control of Rubisco expression by light intensity and several photoreceptors which detect both light quality and quantity have been implicated in the regulation of Rubisco abundance including phytochrome, high irradiance responses, a blue light/UV-A photoreceptor and a UV-B photoreceptor. The mechanisms by which these signals affect Rubisco abundance is varied, involving altered rates of gene transcription (Gallagher and Ellis, 1982), mRNA translation (Berry et al., 1990), post-translational mRNA regulation (Peters and Silverthorne, 1995) and protein turnover (Crafts-Brandner et al., 1996). Moreover, a multigene family, the significance of which has yet to be clarified, encodes the small subunit of Rubisco. While the coding sequence of these individual genes is highly conserved, suggesting little difference in the kinetic properties of the proteins they encode, their expression patterns have shown organ specific differences (Dean et al., 1985; Silverthorne and Tobin, 1990). This could imply that expression of individual genes in different tissues could result in altered responses to environmental signals depending on the precise function of the tissue.

C. Elevated CO₂

In an attempt to understand the effects of predicted increases in atmospheric CO₂ on the carbon exchange in the terrestrial biosphere, many studies have examined the effects of long-term growth at elevated CO₂ on CO₂ assimilation and Rubisco function. Several recent reviews have summarized the present knowledge on Rubisco (Bowes, 1991; Sage, 1994; Woodrow, 1994; Drake et al., 1996). Depending on growth conditions and species, both decreases in Rubisco content per leaf area and/or reductions in Rubisco activation states have been observed. However, in some species no change in either parameter occurs (Drake et al., 1996). It has been frequently pointed out that at elevated CO₂ much less Rubisco per electron transport capacity is required to maintain present ambient CO₂ assimilation rates, particularly at higher temperatures, the effect being less marked at low temperature (Woodrow, 1994; Drake et al., 1996). However there is no evidence that plants are able adjust the balance between Rubisco and electron transport capacity to suit environmental conditions. Experiments with transgenic rice with reduced amounts of Rubisco have demonstrated that plants with 65% of wild-type Rubisco levels were more nitrogen use efficient and may provide a solution for crop growth at elevated CO₂ (Makino et al., 1997b).
In many species gradual decline in assimilation rates are observed after transfer to growth conditions with elevated CO₂ partial pressures. This process has been referred to as a ‘down-regulation’ of the photosynthetic apparatus and is associated with a decline in abundance of photosynthetic proteins including Rubisco (e.g. Besford et al., 1990; Van Oosten and Besford, 1994; Nie et al., 1995; Vu et al., 1997). One explanation for this phenomenon is a feedback regulation of photosynthesis brought about by carbohydrate accumulation within photosynthetic tissues. Several lines of evidence suggest that this the case. Firstly, carbohydrates frequently accumulate in the leaves of plants grown in elevated CO₂ suggesting that there may be a limitation on the ability of sink tissues in some species to utilize the extra photosynthate produced. Secondly, manipulation of source/sink ratio either via fruit removal (Wittenbach, 1983; Plaut et al., 1987) or increasing sink strength via leaf thinning (Diethelm and Shibles, 1989) altered the rate of leaf photosynthesis and abundance of Rubisco protein. More recently, fruit removal experiments with soybean have identified an insoluble high molecular weight form of Rubisco that is associated with down-regulation of photosynthesis (Crafts-Brandner et al., 1991). This involves the synthesis of a new protein (Rubisco Complex Protein) that is able to complex and insolubilize Rubisco isolated from a variety of sources (Crafts-Brandner and Salvucci, 1994). Thirdly, restriction of carbohydrate export from leaves via cold-girdling of stems (Krapp et al., 1993), ectopic expression of yeast invertase in the cell wall compartment of tobacco (von Schaewen et al., 1990) or reduction of the phloem-specific sucrose transport activity in transgenic potato plants (Kuhn et al., 1996) all brought about photosynthetic down-regulation including decreased amounts of Rubisco protein. Fourthly, similar photosynthetic down-regulation could be induced by feeding sugars to detached leaves (Krapp et al., 1991) and this involved sugar-mediated repression of photosynthetic gene expression (Jang and Sheen, 1994). Since these initial observations, our knowledge of the genes that are controlled by carbohydrates and the mechanism for carbohydrate control have increased dramatically. These topics are outside the scope of this chapter but are thoroughly reviewed by Koch (1996) and Smeekens and Rook (1997).

V. Rubisco and C₄ Photosynthesis

The C₄ photosynthetic pathway is a CO₂ concentrating mechanism that operates between mesophyll and bundle sheath cells of leaves (Hatch and Osmond, 1976; Hatch, 1987). Rubisco is located solely in the bundle sheath and the reactions of the C₄ cycle act to concentrate CO₂ in this cellular compartment. This enhances RuBP carboxylation while inhibiting Rubisco oxygenation, thus reducing the amount of photorespiration. CO₂ concentrations in the bundle sheath have not been measured directly but have been estimated to be between 10- to 100-fold greater than in the ambient air (Furbank and Hatch, 1987; Jenkins et al., 1989). Since Rubisco of C₄ species operates in a very different environment compared to Rubisco of C₃ species the regulation of amount and activity may be different.

A. Rubisco Content and CO₂ Assimilation Rate

Similar to photosynthesis in C₃ species, there is a strong correlation between Rubisco activity (or content) and CO₂ assimilation rate, measured at high irradiances (Fig. 15; Usuda, 1984; Hunt et al., 1985; Sage et al., 1987; Furbank et al., 1996; von Caemmerer et al., 1997). Because Rubisco operates close to CO₂ saturation, the relationship between maximal Rubisco activity and CO₂ assimilation rate should be close to 1:1. In transgenic Flaveria bidentis where amounts of Rubisco were reduced via antisense without concomitant changes in the amounts of other photosynthetic enzymes, the decline in CO₂ assimilation rate was not as great as the decline in Rubisco content. This may indicate that in wildtype plants other enzymes like pyruvate Pi dikinase may be limiting the rate or that there may be a small increase in bundle-sheath CO₂ concentrations, as a result of low Rubisco content, and that this enhances Rubisco activity in the antisense plants. Figure 16 shows CO₂ response curves of CO₂ assimilation rates characteristic of C₄ species. Unlike in C₃ species, Rubisco determines the saturated rate of CO₂ assimilation rate whereas the rate of PEP carboxylation determines the initial slope of the response curve and the CO₂ compensation point is very low and largely independent of oxygen concentration.
B. Rubisco Kinetic Constants

The kinetic constants of Rubisco from C₄ species differ from those of C₃ species (Fig. 17; Yeoh et al., 1980, 1981; Jordan and Ogren, 1983; Seemann et al., 1984; Wessinger et al., 1989; Hudson et al., 1990). In particular, the $K_c$ can be several folds higher than in C₃ species. Yeoh et al. (1980) measured the $K_c$ in 60 grass species including 24 C₄ species. They found the $K_c$ for C₃ species to range between 13–26 μM (389–778 μbar) and that of C₄ species between 28–64 μM (838–1916 μbar). In C₄ species that have

*Fig. 15. CO₂ assimilation rate and the ratio of Rubisco to soluble protein versus Rubisco site content in the C₄ species Flaveria bidentis (●, wild-type and ○ transgenic F. bidentis with reduced amounts of Rubisco). Measurements were made at an ambient CO₂ concentration of 350 μbar, an irradiance of 2 mmol quanta m⁻² s⁻¹ and a leaf temperature of 25 °C. Data are redrawn from von Caemmerer et al., 1997.*

*Fig. 16. CO₂ response curves of CO₂ assimilation rate in the C₄ species Flaveria bidentis (closed symbols, wild-type and open symbols, transgenic with a 40 % reduction in Rubisco content). Measurements were made at an irradiance of 2 mmol quanta m⁻² s⁻¹ and a leaf temperature of 25 °C. Data are redrawn from von Caemmerer et al., 1997.*

*Fig. 17. Distribution of Michaelis-Menten constant for CO₂, $K_c$ for different species. Data are replotted from Yeoh et al. (1980, 1981).*

PS II activity in the bundle sheath, bundle sheath O₂ concentrations can be greater than ambient concentrations (Raven, 1977; Berry and Farquhar, 1978) and the apparent $K_m$ (CO₂) in the presence of O₂ could be more than double the above values. If C₄ species evolved from C₃ ancestors with Rubisco with a high affinity for CO₂, this trait was apparently
readily lost with the development of a CO₂ concentrating mechanism. Seemann et al. (1984) also showed that the high $K_c$ is associated with a higher catalytic turnover rate (~1.2 times that of C₃ Rubisco). The extensive measurements of the $K_c$ in C₄ species are not matched by extensive measurements of the relative specificity, $S_{e/o}$, or the $K_o$. In the few C₄ species examined, $S_{e/o}$ is very similar to that of C₃ species (Jordan and Ogren, 1981; Kane et al., 1994). Where measured, the $K_o$ is also greater than the C₃ values, however more detailed measurements of Rubisco kinetic constants of C₄ species are required (Table 4; Badger et al., 1974; Jordan and Ogren, 1981).

**C. Rubisco Activity and Carbamylation**

One might expect that the high CO₂ partial pressures occurring in the bundle sheath may themselves promote full carbamylation, however C₄ species are also known to have activase (Salvucci et al., 1987) and the requirement for the removal of RuBP and other phosphorylated inhibitors apparently remains. The nocturnal inhibitor CA1P has been detected in a number of C₄ species (Vu et al., 1984; Servaites et al., 1986; Moore et al., 1991). However, interesting differences to C₃ species exist. For example in some C₄-species (e.g. Zea mays) the degree of dark inactivation is very much less than that of C₃ species (Usuda, 1985, 1990; Sage and Seemann, 1993) and not all C₄ species show light modulated variations in Rubisco carbamylation (Sage and Seemann, 1993).

**VI. Rubisco and CAM Photosynthesis**

In plants with crassulacean acid metabolism (CAM) photosynthesis is compartmented into four distinct phases over a diel course (Osmond, 1978). The CAM photosynthetic pathway is a temporal CO₂ concentrating mechanism with atmospheric CO₂ fixed nocturnally through PEP carboxylase and the reduced product, malic acid, stored in the vacuole overnight (phase I). A second phase of atmospheric CO₂ uptake (phase II) may occur at dawn, which represents the transition from PEP carboxylase (C₄) to Rubisco (C₃) carboxylation (Osmond and Björkman, 1975; Osmond, 1978; Griffiths et al., 1990). During phase III, stomata close and malic acid efflux from the vacuole and decarboxylation of malate generates a high intercellular partial pressure of CO₂, which may approach 40 mbar (Cockburn et al., 1979; Spalding et al., 1979), while CO₂ is re-fixed through Rubisco. Following decarboxylation the stomata may re-open in the afternoon, and phase IV reflects the shift from C₄ to C₃ atmospheric CO₂ fixation. The CAM pathway permits flexibility in response to environmental stimuli. For example, the transitional

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_c$ (µM)</th>
<th>$K_c$ (µbar)</th>
<th>$K_o$ (µM)</th>
<th>$K_o$ (mbar)</th>
<th>$K_o(1+O/K_o)$ (µbar) at 200 mbar O₂</th>
<th>$V_{o_{max}}/V_{c_{max}}$</th>
<th>$S_{e/o}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atriplex spongiosa</td>
<td>27</td>
<td>814</td>
<td>710</td>
<td>563</td>
<td>1103</td>
<td>0.14</td>
<td></td>
<td>Badger et al., 1974</td>
</tr>
<tr>
<td>Zea mays (exp1)</td>
<td>33</td>
<td>978</td>
<td>550</td>
<td>436</td>
<td>1427</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zea mays (exp2)</td>
<td>28</td>
<td>838</td>
<td>610</td>
<td>484</td>
<td>1184</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zea mays</td>
<td>34</td>
<td>1018</td>
<td>810</td>
<td>643 b</td>
<td>1062</td>
<td>0.3</td>
<td>78</td>
<td>Jordan and Ogren, 1981</td>
</tr>
<tr>
<td>Amaranthus hybridus</td>
<td>16</td>
<td>480</td>
<td>640</td>
<td>508 b</td>
<td>669</td>
<td>0.38</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Kalanchoe diagremontiana (CAM)</td>
<td>14</td>
<td>420</td>
<td>490</td>
<td>389</td>
<td>591</td>
<td>0.24</td>
<td></td>
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</table>

All values were measured at 25 °C. To convert $K_c$ and $K_o$ values from concentration to partial pressures the solubilities for CO₂ of 0.0334 mol (1 bar)⁻¹ and for O₂ of 0.00126 mol (1 bar)⁻¹ were used. $K_c$ values by Badger et al. (1974) where measured in 100 mM Hepes pH 8.3. The values had been calculated with a pKₐ = 6.37 and where recalculated here with a pKₐ = 6.12. Measurements by Jordan and Ogren (1981), were made with 50 mM Bicine buffer pH 8.3 and a pKₐ = 6.23 was used.

⁴ measured as K(O₂)
⁵ measured as K(CO₂)
⁶ $S_{e/o}$ values by Jordan and Ogren (1981,1984) were derived from simultaneous measurements of carboxylation and oxygenation.
phases of daytime CO₂ uptake (II and IV) may be lost in response to drought stress and following prolonged drought, the stomata may remain closed over the entire 24 h period (Kluge and Ting, 1978; Martin, 1996). Under well watered conditions, on the other hand, additional atmospheric CO₂ fixation can occur via C₃ photosynthesis during the transitional phases (II and IV), and this may constitute a significant proportion of carbon gain, in addition to that fixed at night (Borland and Griffiths, 1996). CO₂ fixation via Rubisco is associated with a larger discrimination against ¹³CO₂ compared to ¹²CO₂ than is CO₂ fixation via PEP carboxylase. Borland and Griffiths (1996, 1997) have used combined measurements of carbon isotope discrimination and CO₂ assimilation to assess the amount of PEP carboxylation versus Rubisco carboxylation occurring during phase II and IV.

Rubisco is a substantial component of the proteins in CAM plants exceeding PEP carboxylase in amount and in maximal activity (Cluge and Ting, 1978; Israel and Nobel, 1994, 1995). In Opuntia fusicus-indica PEP carboxylase and Rubisco correspond to 10 and 30% of extractable soluble protein (Israel and Nobel, 1995), the Rubisco value being typical of that found in C₃ species. The ratios of extractable maximal PEP carboxylase to maximal Rubisco activity are usually one to one or less (Israel and Nobel, 1994, 1995,) which contrasts with the C₄ photosynthetic pathway where an excess of PEP carboxylase activity is essential for the generation of elevated bundle sheath CO₂ concentrations.

There are only very few measurements of Rubisco kinetics constants for CAM plants. Although like C₄ species CAM species have evolved from C₃ species and have developed a CO₂ concentrating mechanism, the kinetic constants measured are C₃ like (Table 4; Badger et al., 1975; Yeoh et al., 1981). The CAM pathway requires no special regulation of Rubisco activity. Vu et al. (1984) and Servaites et al. (1986) identified several CAM plants with very low nocturnal Rubisco activity indicative of the presence nocturnal inhibitors. Nocturnal inhibition of Rubisco is however not a general feature of CAM metabolism (Moore et al., 1992; Osmond et al., 1996).

**VII. The Role of Rubisco in C₃-C₄ Intermediates**

Over 20 species of plants exhibit photosynthetic characteristics that are intermediate between C₃ and C₄ plants in that they show reduced photorespiratory CO₂ release and CO₂ compensation points in the range of 7–30 μbar (Edwards and Ku, 1987; Rawsthorne, 1992). All of these plants show a degree of Kranz anatomy, but interveinal distances are much greater than in species. Fig. 18 shows a schematic summary of C₃-C₄ pathways but it is important to keep in mind that the biochemistry of fixation varies among C₃-C₄ species. In all cases Rubisco is present in both mesophyll and bundle-sheath cells, but activities of pathway enzymes in both mesophyll and bundle sheath PCO cycles and in some species, additional CO₂ is supplied via a C₄ cycle.

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The quantitative feasibility of such localized photorespiratory CO₂ release accounting for the observed CO₂ exchange characteristics of C₃-C₄ intermediates was demonstrated by the model of von Caemmerer (1989). The recycling of photorespiratory CO₂ cannot result in a large increase in photosynthetic
rate at ambient CO₂ concentration and at most 15% of leaf Rubisco might be allocated to the bundle sheath for such recycling purposes. However it may be advantageous when stomata restrict CO₂ supply or at high temperatures when photorespiratory rates increase (Monson, 1989; von Caemmerer, 1992).

Some C₃-C₄ intermediates of Flaveria show appreciable C₄ cycle activity (Monson et al., 1986; Dai et al., 1996). However, all of the Rubisco’s of C₁-C₄ intermediates that have been examined have C₃-like kinetic constants (Jordan and Ogren, 1981; Yeoh et al., 1980; Hudson et al., 1990; Wessinger et al., 1989). Little is known about regulation of Rubisco activity in these species.

**VIII. Conclusion**

There have been considerable advances during the last ten years in our knowledge of Rubisco physiology; we now have a working model of Rubisco regulation by Rubisco activase and how this is integrated with the variety of tight binding inhibitors. Transgenic plants have allowed direct quantification of the control that Rubisco exerts on photosynthesis in a variety of environmental conditions confirming and improving our understanding of the major role played by this enzyme in photosynthetic metabolism.

Many signals (e.g. light quality and quantity, carbohydrates, nitrate and cytokinins) that alter the abundance of Rubisco have been identified. However more information is required to understand how these processes are integrated. Increasing advances in plant molecular biology will allow progress in our knowledge of the spatial and temporal signal transduction pathways that coordinate the amount and activity of Rubisco with the growth and development of the plant.

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Photorespiration

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Summary

Photorespiration results in the light-dependent uptake of $\text{O}_2$ and release of $\text{CO}_2$. Oxygenation of Ribulose-1,5-bisphosphate, an unavoidable process, leads to the production of glycolate-2-P. The recycling of glycolate-2-P into glyceraldehyde-3-P via the photorespiratory cycle (C2 cycle) requires a large machinery, consisting of more than 15 enzymes and translocators, distributed over three different organelles, i.e. the chloroplast, peroxisome and mitochondrion. Complex compartmentation is an essential trait of photorespiration. The most fascinating reaction in the C2 cycle occurs in the mitochondria when glycine molecules formed in the peroxisomes are broken down by a complex of proteins (H-, P-, T- and L-proteins) which by concerting their activities, catalyze the oxidative decarboxylation (glycine decarboxylase) and deamination of glycine with the formation of $\text{NADH}$ and the concomitant reduction of $\text{NAD}^+$ to $\text{NADH}$. The remaining carbon of glycine is transferred to tetrahydropteroylpolyglutamate ($\text{H}_4\text{PteGlu}_n$ or folate) to form $\text{CH}_2\text{H}_4\text{PteGlu}_n$. The H-protein plays a pivotal role in the complete sequence of reactions since its prosthetic group (lipoic acid) interacts successively with the three other components of the complex and undergoes a cycle of reductive methylamination, methylamine transfer and electron transfer. The availability of folate to glycine decarboxylase and its recycling through serine hydroxymethyltransferase (SHMT) reaction is a critical step for glycine oxidation during photorespiration. Numerous shuttles exist to support transamination, ammonia refixation and the supply or export of reductants.

I. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is present at a tremendous concentration in the stroma of the chloroplasts (0.2 g·ml⁻¹; Rubisco accounts for up to 30% of total nitrogen in a typical C₃ leaf), is a bifunctional enzyme, catalyzing both the carboxylation and the oxygenation of ribulose-1,5-bisphosphate (RuBP) (Lorimer, 1981). The two reactions involve the competition of molecular CO₂ with O₂ for the enediol form of RuBP which is first generated at the active site of the enzyme. Once the enediol has reacted with either of the gases, the enzyme is committed to form products (Pierce et al., 1986; Bainbridge et al., 1995; Gutteridge and Gatenby, 1995; Cleland et al., 1998). The partitioning of RuBP between carboxylation and oxygenation is dependent on the kinetic parameters of Rubisco. The carboxylation of RuBP results solely in the formation of glycerate-3-P. While five-sixths of glycerate-3-P molecules thus formed are used for regeneration of RuBP, the remaining one-sixth is either exported from the chloroplasts as triose phosphate for synthesis of sucrose and other products in the cytosolic compartment or metabolized to form starch within the chloroplasts. Oxygenation of RuBP leads to the production of one molecule of glycerate-3-P and one molecule of glycolate-2-P (a two carbon compound). Glycolate-2-P, which is formed under ambient conditions at very high rates, is salvaged in the photorespiratory pathway (C₃ cycle) without the net synthesis of triose phosphate. C₃ cycle and the C₄ cycle operate, therefore, in perfect synchronization. In leaves under ambient conditions the rate of oxygenation to carboxylation has been estimated to be as high as 0.4 (Sharkey, 1988). Low intercellular concentrations of CO₂, as may occur, for example, under water stress (e.g. whenever stomata are closed) can result in even higher ratios. Conversely, doubling the CO₂ concentration increases the ratio of Rubisco carboxylation/oxygenation activities by approximately two-fold, reducing photorespiration by about 50% (Sharkey, 1988).

The reaction sequence of the C₃ cycle, has been primarily resolved by Edward Tolbert and collaborators (Tolbert, 1980). Very detailed reviews of the photorespiratory pathway have been published by Chollet and Ogren (1975), Lorimer and Andrews (1981), Huang et al. (1983), Ogren (1984) and Husic et al. (1987). Some of the key regulatory properties of the photorespiratory cycle have been carefully reviewed together with the regulatory interactions that might occur between photorespiration and the Benson-Calvin cycle by Leegood et al. (1995). The value of numerous mutant plants (Hordeum, pea, and Arabidopsis thaliana) in the exquisite elucidation of the mechanism of photorespiration and its relationships with CO₂ fixation and amino acid metabolism has been highlighted by Somerville (1982), Somerville and Ogren (1982) and Blackwell et al. (1988). The mutants were unable to survive in air, but could thrive in atmospheres containing a high concentration of CO₂ (or low [O₂]).

II. The Photorespiratory Pathway

Glycolate-2-P formed in the chloroplasts is hydrolyzed to glycolate, as catalyzed by phosphoglycolate phosphatase present in the chloroplast stroma (Fig. 1). In tobacco this enzyme was found to be a dimer with a subunit size of about 32 kDa (Hardy and Baldy, 1986; Belanger and Ogren, 1987).
The enzyme is saturated at very low glycolate-2-P concentrations ($K_m < 100 \mu M$), which enables a very efficient hydrolysis of the glycolate-2-P formed by the oxygenase reaction (Husic et al., 1987). In mutants lacking phosphoglycolate phosphatase, characterized by Somerville and Ogren (1979), photosynthetic CO$_2$ assimilation was very rapidly inhibited upon exposure to air.

The glycolate is exported from the chloroplasts in exchange for glycerate (Fig. 1). This exchange is catalyzed by a translocator having similar affinities for glycolate, glyoxylate, D-glycerate and D-lactate (Howitz and McCarty, 1985a,b). By this translocator, glycolate and glycerate can be either transported together by counter exchange for each other, or individually by H$^+$ symport (or OH$^-$ antiport) (Howitz and McCarty, 1991). This flexibility allows that the amount of glycerate returning to the chloroplasts to be only half of the amount of glycolate released from the chloroplasts. In addition, the activity of the glycolate-glycerate translocator appears to be high enough to account for in vivo photorespiratory carbon fluxes (Howitz and McCarty, 1985a,b).

The glycolate has to enter the peroxisomal
compartment (peroxisomes are bounded by only a single membrane) to be further metabolized (Fig. 1). The uptake of metabolites into the peroxisomes proceeds via pores (see below), probably formed by a porin present in the peroxisomal membrane. In the peroxisomal matrix the glycolate is oxidized by \( O_2 \) to glyoxylate, as catalyzed by glycolate oxidase, with the concomitant reduction of the flavin mononucleotide (FMN) prosthetic group. This enzyme with a \( K_m \) for glycolate of 0.25–0.4 mM (Husic et al., 1987) catalyzes an irreversible reaction. FMN is reoxidized by oxygen to produce hydrogen peroxide which is, in turn, decomposed by catalase, a heme-containing enzyme. Most of the hydrogen peroxide is degraded by matrix catalase, but the high \( K_m \) (mMolar range) for the enzyme could result in low residual concentrations diffusing into contact with the inner surface of the limiting peroxisomal membrane which contains an ascorbate peroxidase (Bunkelmann and Trelease, 1996) functioning as a peroxide scavenger (Asada, 1992). Glycolate oxidase [a tetramer or other oligomer with subunits of 37–40 kDa (Hall et al., 1985, Volokita and Somerville, 1987)] is one of the very few peroxisomal enzymes for which a high resolution crystal structure is available (Lindqvist et al., 1991). The enzyme crystallizes in an octameric form and the subunit contains a eight-fold beta/alpha-barrel motif corresponding to the FMN domain which is also found in other FMN-dependent enzymes. The apoprotein has a different structure from the holoprotein (Sandalova and Lindqvist, 1993) indicating that the cofactor plays a determinant structural role. The amino acids involved in the structure of the active site have been studied (Stenberg et al., 1995). In addition, the signal for targeting glycolate oxidase into the plant peroxisomes resides on the mature polypeptide and its N-terminal 59 amino acids are dispensable for protein import in an ATP-dependent and temperature-dependent manner (Horng et al., 1995; Volokita, 1991). Indeed peroxisomes do not possess DNA or a protein-synthesizing machinery and acquire proteins by import from the cytosol, usually without proteolytic processing (Lazarow and Fujiki, 1985).

The structure of catalase is far from being elucidated. Generally catalase from plant tissues is composed of four heme-containing subunits with a MW between 54 and 59 kDa. In many plants, catalase exists in multiple forms (Scandalias, 1994; Havir et al., 1996) and little is known about the molecular basis of catalase heterogeneity. In addition, assembly of catalase holoenzymes has not been widely studied (Kunce and Trelease, 1986; Redinbaugh et al., 1990; Banjoko and Trelease, 1995). Transgenic tobacco with 0.05 to 0.15 times the catalase activity of wild type has been reported (Chamnongpol et al., 1996), and it was shown that under high photosynthetic conditions necrotic lesions were produced in leaves owing to dramatic accumulation of \( \text{H}_2\text{O}_2 \).

The glyoxylate is converted in the peroxisomes by transamination to glycine in two alternative ways, as catalyzed by serine:glyoxylate amino transferase (SCAT) and glutamate:glyoxylate amino transferase (GGAT), normally operating in a 1:1 ratio (Fig. 1). A detailed examination of the transaminases in spinach leaf peroxisomes has been made (Rehfeld and Tolbert, 1972). Both amino transferases contain pyridoxal 5'-phosphate (PLP) as cofactor. In contrast with the animal proteins, which usually release their cofactor during the purification procedure, the PLP in plant amino transferases is generally very tightly bound to the enzyme (Givan, 1980). SGAT probably occurs as a dimer of different subunits of 45 and 47 kDa (Hondred et al., 1987). However, despite the fact that the enzyme has been partially purified from several plants, its quaternary structure is not well defined (Noguchi and Hayashi, 1980; Ireland and Joy, 1983; Hondred et al., 1987). Serine is the preferred amino donor (with \( K_m \) values between 0.6 and 2.7 mM) and glyoxylate is the preferred amino acceptor (with \( K_m \) values between 0.15 and 4.6 mM) (Nakamura and Tolbert, 1983). In contrast to GGAT, which like other amino transferases catalyzes a reversible reaction, the conversion of glyoxylate to glycine catalyzed by SGAT is virtually irreversible. GGAT [molecular weight of the native enzyme in spinach 98 kDalton (Noguchi and Hayashi, 1981)] probably also consists of two subunits. Again, the quaternary structure of the enzyme is not well defined. While SGAT is relatively specific for serine as the amino donor, GGAT is less specific. It also reacts with alanine as amino donor and with 2-oxoglutarate as acceptor (Noguchi and Hayashi, 1981; Ta and Joy, 1986). The \( K_m \) values for glyoxylate and the amino donor (glutamate or alanine) are 0.15 and 2–3 mM respectively. Interestingly decarboxylation of glyoxylate readily occurs in Arabidopsis and barley mutants lacking key enzymes involved in photorespiration such as serine hydroxymethyltransferase (Somerville and Ogren, 1981) and SGAT (Murray et al., 1987). Likewise decarboxylation of glyoxylate...
also occurs in leaves treated with phosphinothricin, a potent inhibitor of glutamine synthetase (Wendler et al., 1992). Glyoxylate can be metabolized, therefore, by reactions other than transamination. However, as demonstrated by Somerville and Somerville (1983), the preferred route of glyoxylate metabolism during the course of photorespiration is amination rather than conversion to formate and CO₂.

The glycine thus formed is released from the peroxysome via pores and taken up into the mitochondria to be oxidized (Fig. 1). The most fascinating reaction in the C₂ cycle occurs in the mitochondria when glycine molecules formed in the peroxisomes are broken down by a complex of proteins (glycine decarboxylase or glycine cleavage) which byconcerting their activities, catalyse the oxidative decarboxylation and deamination of glycine with the formation of CO₂, NH₃ and the concomitant reduction of NAD⁺ to NADH (Douce and Neuburger, 1989; Rawsthorne et al., 1995). The remaining carbon, the methylene carbon of glycine, is then transferred to 5,6,7,8-tetrahydropteroylpolyglutamate (H₄PteGluᵦ), oligo- (Imeson et al., 1990) to form N₅,N¹⁰-methylene-5,6,7,8-tetrahydropteroylpolyglutamate (CH₂H₄PteGluᵦ) (Fig. 2). The latter compound reacts with a second molecule of glycine in a reaction catalysed by serine hydroxymethyltransferase (SHMT) to form serine (Douce and Neuburger, 1989; Rawsthorne et al., 1995) (Fig. 2). In the course of glycine decarboxylation and deamination one molecule of serine leaves the mitochondrion, and two molecules of glycine are taken up. Despite the evidence that glycine and serine transporters are common in a number of

*Fig. 2. Schematic representation of the glycine oxidation in green leaf mitochondria. During photorespiration glycine is cleaved in the matrix space by the glycine cleavage system containing four protein components (named P-protein, H-protein, T-protein and L-protein) to CO₂, NH₃ and N₅,N¹⁰-methylene-5,6,7,8-tetrahydropteroylpolyglutamate (CH₂H₄PteGluᵦ). The latter compound reacts with a second molecule of glycine to form serine and 5,6,7,8-tetrahydropteroylpolyglutamate (H₄PteGluᵦ) in a reaction catalyzed by serine hydroxymethyltransferase (SHMT). H-protein plays a pivotal role in the complete sequence of reactions since its prosthetic group, 5[3-(1,2-dithiolanyl]pentenoic acid (lipote acid), interacts successively with the three other components of the complex and undergoes a cycle of reductive methylation, methylamine transfer and electron transfer. NADH thus produced is oxidized either by the respiratory chain tightly coupled to the synthesis of ATP or by oxaloacetate, owing to the malate dehydrogenase located in the matrix space working in the reverse direction. A rapid malate-oxaloacetate transport shuttle appears to play an important role in the photorespiratory cycle in catalyzing the transfer of reducing equivalents generated in the mitochondria during glycine oxidation to the peroxisomal compartment in the reduction of hydroxypropyruvate. Note the unusual stoichiometry of two glycine molecules entering the mitochondrial matrix in exchange for one serine leaving.*
biological systems, there is no consensus that the much larger flux of glycine across the inner membrane is carrier-mediated. Indeed, according to several groups influx of glycine is not related to the energy status of the mitochondrion and appears diffusional because transport rates increase linearly up to very high glycine concentrations (for a review, see Douce and Neuburger, 1989). This would allow a flexible stoichiometry for the exchange reaction that would accomodate the 2:1 ratio needed for the reaction by which glycine is metabolized. Nevertheless, because of the very high flux through the photorespiratory pathway one would expect that the transfer of glycine and serine is translocator-mediated, and there are indeed experimental results which suggest that such translocators may exist in plant mitochondria (Walker et al., 1982; Yu et al., 1983; Oliver 1987; Douce and Neuburger, 1989). For the present, the details of glycine and serine transport in green leaf mitochondria remain a mystery, and more work is needed to confirm a mechanism. The question whether both glycine and serine are transported by a single protein or by two different ones cannot be answered at present.

Serine released from the mitochondria enters the peroxisomal matrix, where it is converted to hydroxypyruvate by the above mentioned serine:glyoxylate amino transferase (Fig. 1). Since mitochondrial oxidation of two molecules of glycine yields one molecule of serine, only half of the glyoxylate can be transaminated by serine, and therefore glutamate:glyoxylate amino transferase is required for transamination of the remaining second half of the glyoxylate. Since only serine:glyoxylate amino transferase catalyzes an irreversible reaction, serine is a preferential amino donor over glutamate for glyoxylate transamination, ensuring that the serine released from the mitochondria is further metabolized in the peroxisomes. Thus the conversion of serine to hydroxypyruvate is almost unidirectional, which strongly suggests that photorespiratory serine does not exert a feed-back control of its own synthesis. Indeed the mitochondrial concentration of serine is a key regulatory point of the glycine cleavage system [serine binds to the P-protein in a manner that is competitive with the binding of glycine \( K_m \text{glycine} = 6 \text{mM} \); \( K_i \text{serine} = 4 \text{mM} \)] (see below) and that the rate of serine export is a critical step for glycine oxidation. Interestingly, Somerville and Ogren (1980), Murray et al. (1987) and McHale and Zelitch (1988) demonstrated that mutations blocking serine:glyoxylate aminotransferase in various plants are conditionally lethal, permitting normal growth in a CO₂-enriched atmosphere, but converting photorespiration into a lethal process in air.

In the peroxisomes, hydroxypyruvate is reduced by NADH to glycerate in a reaction catalyzed by hydroxypyruvate reductase (Fig. 1). The enzyme has been purified to homogeneity from greening cucumber cotyledons and spinach leaves and was found to occur as a dimer consisting of subunits of 41–47 kDalton (Kohn et al., 1970; Titus et al., 1983; Greenler et al., 1989; Givan and Kleczkowski, 1992). The enzyme displays a high preference for NADH \( K_m = 6 \mu \text{M} \) rather than NADPH. The peroxisomal hydroxyypuvrate reductase has also been named glyoxylate reductase, since this enzyme also catalyzes the reduction of glyoxylate. This probably does not play any role under physiological conditions, however, since the \( K_m \) of the enzyme for glyoxylate (5–15 mM) is two orders of magnitude higher than the \( K_m \) for hydroxypyruvate (62–120 \( \mu \text{M} \)) (for ref. see Husic et al., 1987). Estimation of the equilibrium constant \( K_{eq} = [\text{D-glyceric acid}] \text{NAD/}[\text{hydroxypyruvic acid}] \text{NADH}] = 1.6 \times 10^{12} \text{M}^{-1} \) indicates that the reaction strongly favors glycerate production that is the forward motion of the photorespiratory cycle. NADH is generated in peroxisomes through a specific malate dehydrogenase activity. The work of Kim and Smith (1994) suggests that there is a single gene encoding glyoxysomal and peroxisomal malate dehydrogenase. Leaves also contain a powerful cytosolic hydroxypyruvrate reductase activity preferring NADPH to NADH as a cofactor (Kleczkowski and Randall, 1988; Givan and Kleczkowski, 1992). This reductase could use glyoxylate as an alternative substrate. This enzyme is not believed to be involved in the main route of carbon flow through the glycolate pathway. It could serve as backup reaction utilizing hydroxypyruvate and/or glyoxylate leaked from the peroxisomes to the cytosol (Kleczkowski et al., 1990). Presumably, NADPH is generated in the cytosol through the cytosolic oxidative pentose-phosphate pathway. Convincing evidence that cytosolic hydroxypyruvate reductase can play a role in photorespiration come from barley mutants lacking the peroxisomal NADH-dependent hydroxypyruvate reductase: in these plants hydroxypyruvate reduction proceeds, very likely, in the cytosolic compartment (Murray et al., 1989).

The glycerate formed from hydroxypyruvate reduction leaves the peroxisomes and is transported
to the chloroplast stroma, where it is phosphorylated in an almost irreversible reaction to 3-phosphoglycerate \( (K_{eq} = 300) \) by glycerate kinase (Fig. 1) (Kleczkowski et al., 1985). The enzyme purified from leaves is a monomer of about 40 kDa (Schmidt and Edwards, 1983) and displays \( K_m \) values of 0.25 mM and 0.21 mM for glycerate and ATP respectively (Kleczkowski et al., 1985). Its kinetic properties are consistent with a random sequential binding of the substrates. Glycerate kinase activity exceeds the maximal rate of glycerate transport and is, therefore, probably not a limiting step of the photorespiratory cycle (Husie et al., 1987).

In a plant cell, chloroplasts and mitochondria are mostly in close proximity to each other. The \( \text{NH}_4^+ \) (and or \( \text{NH}_3 \)) released by mitochondrial oxidation of glycine passes through the inner membrane of mitochondria and chloroplasts. Whether this passage occurs by simple diffusion, or is brought about by specific ion channels or translocators [a gene from Arabidopsis has recently been identified for a high affinity ammonia transporter which is expressed as a transcript in roots(Ninnemann et al., 1994)] is still a matter of debate. Likewise one of the major unresolved aspects of the inner membranes of mitochondria and chloroplasts in all eukaryotes concerns the \( \text{CO}_2 \) permeability of the membranes. In other words it is not known which Carbon inorganic species\( (\text{CO}_2, \text{HCO}_3^-) \) is transported in cell organelles. In this connection Rolland et al (1997) using a mutant of Chlamydomonas reinhardtii have suggested the existence of a specific system within the plastid envelope which promotes efficient inorganic carbon uptake into chloroplasts.

The refixation of \( \text{NH}_3 \) in the chloroplasts proceeds in the same way as in nitrate assimilation (Lea et al., 1978; Givan et al., 1988). In mesophyll cells \( \text{NH}_4^+ \) reacts with glutamate and ATP to form glutamine and ADP, as catalyzed by glutamine synthetase, followed by glutamate synthase catalyzing the reaction of glutamine with 2-oxoglutarate with reduction by ferredoxin to two molecules of glutamate. One molecule of glutamate thus formed is exported to the peroxisomes as amino donor for glutamate:glyoxylate amino transferase in exchange for 2-oxoglutarate. The chloroplast is the sole location for glutamine synthetase activity in mesophyll cells. Likewise the ferredoxin-dependent glutamate synthase is exclusively located in the chloroplasts of \( \text{C}_3 \) and \( \text{C}_4 \) leaves (Becker et al., 1993; Lea and Forde, 1994). Mutants of Barley deficient in glutamine synthetase (Blackwell et al., 1987; Wallsgrove et al., 1987) accumulate high amounts of ammonia in the leaves when placed in air owing to the rapid conversion of glycine to serine during the process of photorespiration in the mitochondria. On the other hand, mutants of ferredoxin-dependent glutamate synthase of various plants including Arabidopsis, barley and pea (for a review see Lea and Forde, 1994) accumulate glutamine instead of ammonia. The careful analysis of all these mutants has confirmed the pathway of ammonia assimilation by the glutamate synthase cycle (Lea et al., 1978) and not via glutamate dehydrogenase. It is clear, therefore, that during the course of photorespiration, 2-oxoglutarate is massively imported into the chloroplast, and glutamate, deriving from the glutamine synthetase/glutamate synthase cycle, is exported towards the peroxysome. Two different dicarboxylate antiport systems with overlapping substrate specificities are involved in this process. The 2-oxoglutarate/malate translocator imports 2-oxoglutarate in exchange for stromal malate whereas export of glutamate from the chloroplast in exchange for malate is catalyzed by the glutamate/malate translocator. Malate is the counterion for both translocators, resulting in 2-oxoglutarate/glutamate exchange without net malate import (Woo et al., 1987; Flügge et al., 1996) (Fig. 1). Weber et al. (1995) have determined the nucleotide sequence of a cDNA clone encoding the spinach chloroplast 2-oxoglutarate/malate translocator and its deduced amino acid sequence. The translocator contains a 12-helix motif and probably functions as a monomer. Surprisingly all other organellar translocators known so far, including those from mitochondria, belong to a distinct group of transporters that have 5–7 transmembrane helices functioning as dimers. The transit peptide of this translocator is extremely long and differs from those of other inner envelope membrane proteins but its import characteristics closely resemble those described for other inner envelope membrane proteins. Expression of the coding region of this translocator in yeast cells and subsequent reconstitution of the recombinant protein in liposomes revealed that this translocator mediates the exchange of 2-oxoglutarate with malate.

III. Glycine Oxidation

Rapid glycine oxidation which requires the
functioning of two enzymatic complexes (glycine decarboxylase and serine hydroxymethyltransferase) working in concert has been shown to be exclusively present in mitochondria from leaf tissues. It is a key step of the photorespiratory cycle because it results in the conversion of a two-carbon molecule into a three-carbon molecule that, thereafter, could be reintroduced in the Benson-Calvin cycle (Walker and Oliver, 1986; Bourguignon et al., 1988). Like its mammalian counterpart (Kikuchi and Hiraga, 1982) it contains four different components designated as the P-protein (a homodimer containing pyridoxal phosphate, 105 kDa), H-protein (a monomeric lipoamide-containing protein, 14 kDa), T-protein (a monomer catalyzing the H$_4$PteGlu$_n$-dependent step of the reaction, 41 kDa), and L-protein (a homodimer containing flavin adenine dinucleotide [FAD] and a redox active cystine residue, 59 kDa). Likewise the glycine decarboxylase complex from plant leaf mitochondria is closely related to similar enzyme complexes found in bacteria such as Pseudomonas glycinophilus and Athrobacter globiformis (Klein and Sagers, 1966; Kochi and Kikuchi, 1969). The primary sequence of all these proteins from different sources were determined by cDNA and genomic cloning. The range of organisms includes Pisum sativum as the first plant species for which acetone extraction (Walker and Oliver, 1986). It has been possible to isolate the individual component proteins and to reconstitute the functional complex that catalyses the conversion of glycine into serine and NADH in the presence of NAD$^+$, H$_4$PteGlu$_n$ and SHMT (Bourguignon et al., 1988). Measurements of the amount of each component protein within the matrix and analysis of the optimal subunit ratios for reconstitution suggest a subunit stoichiometry of four P-protein/27 H-protein/9 T-protein/2 L-protein.

The 13.3 kDa lipoamide-containing H-protein plays a pivotal role in the complete sequence of reactions since its prosthetic group, 5-[3-(1,2-dithiolanyl]pentanoic acid (lipoy acid), interacts successively with the three other components of the complex and undergoes a cycle of reductive methylamination, methylamine transfer and electron transfer (Fig. 2). The lipoyl moiety in the H-protein is attached by an amide linkage to the ε-amino group of a lysine residue (Lysine 63 in the 131 amino acid pea H-protein; Mérand et al., 1993) which is located in the loop of a hairpin configuration (Parès et al., 1994; Cohen-Addad et al., 1995) (Fig. 3). When the complex is diluted it tends to dissociate into its component enzymes and the H-protein acts as a mobile co-substrate that commutes between the other three proteins. The reaction begins with the amino group of glycine forming a Schiff base with the pyridoxal phosphate of the P-protein. The carboxyl group of glycine is lost as CO$_2$ and the remaining methylamine moiety is passed to the lipoamide cofactor of the H-protein (the methylamine intermediate, which is a rather stable structure, can be separated easily from the oxidized H-protein on ion-exchange chromatography; Neuburger et al., 1991). During the course of this reaction the lipoamide-methylamine arm formed rotates to interact readily with several specific amino acid residues located within a cleft at the surface of the H-protein (Fig. 3). Such a situation locks the methylamine group into a very stable configuration within a hydrophobic pocket preventing the nonenzymatic release of NH$_3$ and formaldehyde due to nucleophilic attack by water molecules. In other words the H-protein not only picks up the methylamine group in one active site and delivers it to another active site, but the protein also protects the methylamine group while doing so. The lipoamide-bound methylamine group is shuttled to the T-protein where the methylene carbon is transferred to H$_4$PteGlu$_n$ to produce CH$_2$H$_4$PteGlu$_n$ and the amino group is released as NH$_3$. During the course of this reaction the methylamine group undergoes a nucleophilic attack by the N-5 atom of the pterin ring.
of H₄PteGlu₉. Only the 6S stereoisomer of H₄PteGlu₉, the natural occurring form, is the substrate for this reaction in vivo (Besson et al., 1993). Finally, the L-protein (lipoamide dehydrogenase) catalyzes the regeneration of the oxidized form of lipoamide with the sequential reduction of FAD and NAD⁺. In pea leaf mitochondria, the pyruvate dehydrogenase and glycine decarboxylase complexes share the same dihydrolipoamide dehydrogenase (E3 component of pyruvate dehydrogenase, L-protein of glycine decarboxylase) and this raises some interesting questions about the regulation of synthesis and control of the distribution of this unique enzyme associated with different complexes (Bourguignon et al., 1996). All of the reactions catalysed by the glycine cleavage system are fully reversible. For example, the H- and L-proteins together catalyse the reversible exchange of electrons between NADH and lipoamide bound to the H-protein (Neuburger et al., 1991).

Compounds that react with either the lipoamide cofactor of the H-protein (such as arsenite) or with the pyridoxal phosphate of the P-protein (such as carboxymethoxyamine, methoxylamine and acetlydrazide), strongly inhibit the glycine cleavage system (Sarojini and Oliver, 1985). We believe that molecules acting at the level of the protein components of the glycine decarboxylase complex would exhibit herbicidal potency. Indeed experiments using Arabidopsis showed that in plants deficient in glycine decarboxylase activity, photosynthesis was not impaired in non-photorespiratory conditions, but was irreversibly inhibited in atmospheres that allowed the rapid production of glycolate by chloroplasts (Somerville and Ogren, 1982; Somerville and  

![Fig. 3. Structure of the H-protein from the pea leaf glycine decarboxylase complex. Schematic ribbon representation of the overall folding of the proteins (oxidized form, methylamine-loaded form and apoprotein). The conformational of all these forms are virtually identical, consisting of seven β-strands in a sandwich structure made of two antiparallel β-sheets. Note that the lipoate cofactor attached to a specific lysine side chain is located in the loop of a hairpin configuration but following methylamine transfer it is pivoted to bind into a cleft at the surface of the H-protein. The apoprotein exhibits the same structure than the holoprotein indicating that the cofactor plays no determinant structural role. See also Color Plate 3.](image-url)
Somerville, 1983). Likewise the host-selective toxin victorin [a group of closely related cyclized pentapeptides produced by the fungus *Cochliobolus victoriae*) (Macko et al., 1985)], which severely inhibits glycine decarboxylase activity [it binds strongly to the P- and H-proteins (Wolpert et al., 1994; Navarre and Wolpert, 1995)] causes symptoms typical of victoria blight disease on susceptible plants.

**B. Reaction Catalysed by Serine Hydroxymethyltransferase**

In leaf mitochondria, the major function of SHMT is to recycle $\text{CH}_2\text{H}_4\text{PteGlu}_{n+}$, produced by the T-protein activity, to $\text{H}_2\text{PteGlu}_{n+}$ to allow the continuous operation of the glycine-oxidation reaction (Bourguignon et al., 1988) (Fig. 2). SHMT has been purified from pea leaf mitochondria and is a 220 kDa homotetramer with a subunit molecular weight of 53 kDa (Bourguignon et al., 1988; Turner et al., 1992b; Besson et al., 1995). Each subunit has a single pyridoxal-phosphate bound as a Schiff base to an $\varepsilon$-amino group of a lysine residue (SHMT is yellow, exhibiting an absorption maximum at 428 nm due to the pyridoxal-P bound as a Schiff base) (Schirch, 1984). In plants, the rate constant of serine to glycine conversion by SHMT is about 15 times higher than the rate constant of the reverse direction and this shows that the reaction favors serine to glycine conversion (Besson et al. 1993). It is clear, therefore, that the SHMT reaction must be permanently pushed out of equilibrium, towards the production of serine and $\text{H}_2\text{PteGlu}_{n+}$ to allow the whole process to take place. This was apparently the case because Rébeillé et al. (1994) have observed high $\text{CH}_2\text{H}_4\text{PteGlu}_{n}/\text{H}_2\text{PteGlu}_{n+}$ ratios in the medium during the steady-state course of glycine oxidation. These very high ratios were presumably the result of the highest glycine decarboxylase activity compared with SHMT activity, that they measured in the matrix space. If the glycolate pathway is strictly cyclic as suggested by Lorimer and Andrews (1981), then all $\text{CH}_2\text{H}_4\text{PteGlu}_{n+}$ formed upon glycine oxidation must be used for serine synthesis. In other words, $\text{CH}_2\text{H}_4\text{PteGlu}_{n+}$ arising from glycine during operation of the glycolate pathway does not gain access to the general $\text{C}_1$ pool. Somerville and Ogren (1982) were among the first to show that glycine decarboxylase is not essential under nonphotorespiratory conditions.

The glutamate chain length (one to six glutamate residues) influences the affinity constant for $\text{H}_2\text{PteGlu}_{n+}$ and the maximal velocities displayed by SHMT and T-protein. Thus, the affinity of tetrahydrofolate for the T-protein of the glycine decarboxylase system and SHMT increases considerably with the number of glutamate residues (Besson et al., 1993). These results are consistent with analyses of plant mitochondrial folates which revealed a pool of polyglutamates (approx. 1 nmol/mg protein) dominated by tetra and pentaglutamates which accounted for approx. 25 and 55% respectively of the total pool (Besson et al., 1993). This chain may have a key role in controlling the velocity and the coordination of the two reactions catalysed by glycine decarboxylase and SHMT. Indeed polyglutamates are known to increase the efficiency of sequential folate-dependent enzymes by enhancing the 'channeling' of intermediates between the active sites of protein complexes. The polyglutamates of leaf mitochondria are probably largely bound in a non-tight fashion to the active sites of T-protein and tetrameric SHMT, the two major folate-dependent enzymes (Rébeillé et al., 1994; Prabhu et al., 1996).

On the basis of the molecular masses of these proteins, and taking into account the fact that the tetrameric SHMT bound one folate molecule per subunit and the monomeric T-protein bound one folate molecule, Besson et al. (1993) have calculated that these two proteins were able to bind all the folate of the mitochondria (approx. 1 nmol/mg protein). There is some strong evidence that the negatively charged $\alpha$-carboxyl groups of the poly-$\gamma$-glutamate chain could bind at specific points, such as basic groups of the proteins. In this context, it is notable that regions of the amino acid sequence of the pea T-protein are characterized by the alignment of basic residues (Bourguignon et al., 1993), a favorable condition for ionic interaction with the negatively charged polyglutamate chain. However, a close comparison of the known primary amino acid sequence of the T-protein (Bourguignon et al., 1993) with that of SHMT (Turner et al., 1992b), both enzymes originating from pea leaf mitochondria, did not reveal conserved folylpolyglutamate-binding consensus sequences (Bourguignon et al., 1993). Each folate-dependent enzyme might have its own specific folate-binding site, or the binding sites might rely on secondary or tertiary structures. The results of Rébeillé et al. (1994) also indicated that $\text{H}_2\text{PteGlu}_{n+}$ binding to folate-dependent proteins significantly contributed to the protection of this readily oxidizable compound [for in vitro manipulation this molecule requires strict
anaerobic conditions (Bourgignon et al., 1988)].

C. Metabolic Control of Glycine Oxidation

The rate of glycine release during the course of photorespiration is as much as 50% of the photosynthetic rates of about 3 μmol CO₂ fixed mg⁻¹ chlorophyll min⁻¹ and some five times the rate of normal tricarboxylic acid cycle activity. In order to accomplish rapid rates of glycine oxidation to cope with all the glycine molecules flooding out of the peroxisomes, the glycine decarboxylase system linked to SHMT is present at high concentrations within the mitochondrial matrix (where it comprises about half of the soluble proteins in mitochondria from fully expanded green leaves; 0.2 g per ml of matrix). Such a situation readily explains the relatively lower lipid-to-protein and cytochrome-to-protein ratios in leaf mitochondria compared to those from non-photosynthetic tissues (Gardestrom et al., 1983; Day et al., 1985a). This is in contrast with the situation observed in mammalian mitochondria where glycine decarboxylase represents a minute fraction of the total matrix protein (in humans, its absence due to genetic deficiency leads to a dramatic accumulation of glycine in the blood and to severe neurological diseases).

Glycine is rapidly oxidized by mitochondria from C₃ plant leaves, the rate of glycine oxidation being between 200–400 nmol O₂ consumed min⁻¹ mg⁻¹ of protein (Douce et al., 1977, Day et al., 1985a). The concentration of NAD⁺ within the mitochondrial matrix influences strongly the activity of NAD⁺-linked glycine decarboxylase, with higher concentrations stimulating. This can be altered by adding NAD⁺ to isolated mitochondria owing to the presence of a specific NAD⁺ carrier on the inner mitochondrial membrane (Neuburger et al., 1985). In addition glycine decarboxylase activity is not affected by light, by reversible covalent modification, by control proteins or by proteolytic activation. Its catalytic activity is only regulated by the NADH/NAD⁺ molar ratio which affects the L-protein directly and hence regeneration of the oxidized lipoamide moiety which is bound to the H-protein. Neuburger et al. (1986) have shown that NADH is a competitive inhibitor with respect to NAD⁺ (Kᵣ NAD⁺ = 75 μM; Kᵣ NADH = 15 μM). This means that increasing the ratio of NADH to NAD⁺ in the matrix space will result in a logarithmic increase in inhibition of enzyme activity. Parenthetically, the activity of the pyruvate dehydrogenase complex containing lipoamide dehydrogenase is also regulated by NADH, which is competitive with respect to NAD⁺. Consequently, NADH generated at extremely high rates during glycine oxidation must be reoxidized at equally high rates if photorespiration is to continue (see below).

The glycine metabolism of C₄ plants is almost unknown. This, at least in part, is due to the difficulties in isolating sufficient amount of mitochondria from bundle-sheath cells. However, in plants which use the C₄ dicarboxylic acid pathway, it seems that bundle-sheath cells, in contrast with mesophyll cells (Gardestrom and Edwards, 1985; Ohnishi and Kanai, 1983), have the capacity to decarboxylate glycine at high rates. Indeed, mesophyll cells of C₄ plants also lack the ability to produce glycine via photorespiration because they lack Rubisco (Edwards and Walker, 1983). This unique distribution of photorespiratory enzymes in leaves of C₄ plants has profound implications for leaf metabolism (Cheng et al., 1988).

The distribution of glycine decarboxylase in leaves of Moricandia arvensis (C₃-C₄ intermediate photosynthesis) resembles that in leaves of C₄ species (Rawsthorne, 1992). However the mesophyll cells of Moricandia arvensis, in contrast with the mesophyll cells of C₄ plants, have the capacity to catalyze the photorespiratory pathway at least as far as glycine. Consequently the major site of release of photorespiratory CO₂ in leaves of Moricandia arvensis is in the mitochondria of the inner wall of the bundle-sheath cells. Such a localization greatly facilitates the light-dependent recapture of photorespiratory CO₂ (The bundle-sheath cell mitochondria are in close association with overlying chloroplasts through which the CO₂ must pass).

D. Developmental Biology

Northern and western blot analyses of the expression of the glycine decarboxylase in pea revealed that the P-, H-, and T-proteins were expressed almost exclusively in the leaf tissue and a light-dependent transcriptional control of the genes encoding these proteins was suggested (Kim and Oliver, 1990; Macherel et al. 1990; Kim et al., 1991; Turner et al., 1992a; Bourguignon et al. 1992). From run-on transcription measurements, nuclei isolated from green pea leaves make about eight times more H-protein and P-protein mRNA than do nuclei isolated from etiolated plants (Srinivasan et al., 1992). However, in contrast to these proteins, the expression of the L-protein occurs in all of the tissue examined.
so far because it is also the component of other mitochondrial complexes, in particular the pyruvate dehydrogenase complex (Bourguignon et al., 1996).

All the component proteins of the glycine decarboxylase complex were nuclear-encoded, synthesized on 80S cytosolic ribosomes, and imported into the mitochondria (Srinivasan et al., 1992). The accumulation of glycine decarboxylase proteins in mitochondria from green leaves could be attributed to a light-dependent transcriptional control of the genes encoding these proteins (Srinivasan et al., 1992). The analysis of steady-state levels of rbcS mRNA during pea leaf development revealed a pattern of expression similar to that of the P-, H-, and T-proteins (Vauclare et al., 1996). These results suggest that the genes encoding specific glycine decarboxylase proteins and photosynthetic genes such as rbcS follow a similar transcriptional regulation scheme, as proposed earlier by Kim and Oliver (1990). In fact several light-responsive elements (GT boxes with the GGTTAA consensus core sequence) have been characterized in the promoter region of the gdcH gene from Arabidopsis (Srinivasan and Oliver, 1995). These authors also suggest that more control elements may be responsible for the constitutive low levels of gene expression noted in all non-photosynthetic tissues. In support of this suggestion, two main transcription sites were detected in the gene encoding H-protein (Macherel et al., 1992). However, such a basal expression may occur, but is certainly negligible compared with the very high level of expression in leaves required for photosynthesis.

It is tempting, therefore, to parallel the accumulation of glycine decarboxylase in the matrix of mitochondria with the accumulation of Rubisco in the stroma of the chloroplast because both enzymes reach millimolar concentrations when plants are grown in light conditions. Likewise, mitochondrial SHMT (Turner et al., 1992b), peroxysomal hydroxypyruvate reductase (Bertoni and Becker, 1996), glycolate oxidase (Tsugeki et al., 1993), Ser:glyoxylate aminotransferase (Hondred et al., 1987), and chloroplastic glutamine synthetase (Edwards and Coruzzi, 1989) mRNA accumulations are strongly stimulated by white light. For example, the GUS reporter gene was used to compare the expression patterns of the pea plastidic glutamine synthetase genes in transgenic tobacco plants (Edwards et al., 1990). It was found that the promoter of glutamine synthetase gene conferred light-regulated expression on the GUS gene in photosynthetic cells of leaves, stems and cotyledons. Interestingly, elevated CO₂ was also shown to inhibit the white-light induced accumulation of mRNA for several photorespiratory enzymes including chloroplastic glutamine synthetase (Edwards and Coruzzi, 1989) and peroxysomal hydroxypyruvate reductase (Bertoni and Becker, 1996). Does elevated CO₂ also affect the white-light-induced appearance of P-, T-, and H-mRNAs in dark-adapted leaves to allow fine tuning of the mRNA levels to provide amounts of active glycine decarboxylase appropriate for the current metabolic state?

In monocotyledonous leaves, the P-, H-, and T-proteins of glycine decarboxylase accumulate along the developmental gradient from the young tissue at the leaf base to the older tissue at the tip (Rogers et al., 1991; Tobin and Rogers, 1992). It has been found that photosynthetic and photorespiratory enzymes including glycine decarboxylase proteins, are both located in the matured region of the leaf and follow the same gradient of differentiation and a similar spatial expression. However during the course of pea leaf development, the appearance of glycine oxidation capacity seems to be closely related to the opening of the leaflets, which occurs after the 7-day stage, an event that allows the leaf to function as a morphologically efficient solar captor. This was correlated with the dramatic accumulation of the glycine decarboxylase complex proteins, which was shown to occur in preexisting mitochondria, producing an increase in their density. Thus in the case of young green leaves (7-d-old plants) or etiolated leaves, the bulk of the mitochondria remained in the top part of the self-generated Percoll gradient, whereas the mitochondria isolated from mature leaves (12-d-old plants) were found in the bottom part of the gradient (Vauclare et al., 1996). In this case, and in contrast with monocotyledonous leaves, there was a pronounced lag time between the appearance of Rubisco and glycine decarboxylase that is possibly due to a translational control that is lifted when the leaflets open. Indeed although the mRNAs for both enzymes follow similar patterns during the development of pea leaves, the biosynthesis of Rubisco starts several day before that of glycine decarboxylase. The underlying physiological meaning is that during development, photorespiration appears only after photosynthesis has reached a level of activity that requires high amounts of photorespiratory enzymes to cope with the recycling of two-carbon metabolites.
This implies the existence of post-transcriptional control of gene expression. It may result from some as yet unidentified metabolic signals from fully mature chloroplasts or peroxisomes (glycolate? glycine?). Interestingly Sinclair et al. (1996) have recently shown that glycine induces the expression of the gene encoding the P-protein together with glycine decarboxylase activity in Saccharomyces cerevisiae.

The synthesis of various cofactors including lipoic acid and H₄PteGluₙ should keep pace with the rapid accumulation of glycine decarboxylase and SHMT within the mitochondrial matrix occurring during the course of green leaf development. In this connection, Spronk and Cossins (1972) observed that green cotyledons have much higher folate levels than etiolated leaves of similar ages. Likewise, the biosynthesis of folate compounds in pea seedlings during germination increased more rapidly in light than in dark (Okinaka and Iwai, 1970). Neuburger et al. (1996) calculated that approx. 50% of the total folate pool in pea leaves was associated with the mitochondria whereas only 6–7% was associated with chloroplasts, the remaining 40–45% of the folate pool being presumably associated with the cytosol and/or the nuclei. Considering the small size of the mitochondrial compartment, these organelles have very likely the highest folate concentration. The results of Neuburger et al. (1996) also indicated that higher plant mitochondria are a major site for tetrahydrofolate synthesis. Indeed they observed that all the enzymes required for H₄PteGluₙ synthesis including dihydrotetrahydrofolate synthase, dihydrotetrahydrofolate synthase, dihydrofolate reductase and a folylpolyglutamate synthetase were located in this compartment. In other words plant mitochondria are able to synthesize H₄PteGluₙ from 6-hydroxymethyl-7,8-dihydropterin, p-aminobenzoic acid and glutamate de novo (p-aminobenzoic acid is synthesized from chorismate, a branch point in the aromatic acid pathway which has been localized in plastids and cytosolic compartment. On the other hand there are actually no data available concerning the subcellular localization of 6-hydroxymethyl-7,8-dihydropterin in higher plants). Surprisingly Neuburger et al. (1996) were not able to detect these enzymes in chloroplasts, nuclei or cytosol. This observation, together with the fact that the bifunctional 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydrotetrahydropterate synthase enzyme was synthesized with a putative mitochondrial transit peptide and that a single copy gene was observed in nuclear DNA, strongly suggests that plant mitochondria are, indeed, the unique site of H₄PteGluₙ synthesis in plants (Rebeillé et al., 1997). This raises the question as to whether the metabolic flux through this biosynthetic pathway responds to the dramatic accumulation of folate-dependent enzyme (T-protein and SHMT) observed during the course of leaf development. This is an intriguing question that will need to be addressed. Such a localization also raises the problem of folate transport through the inner mitochondrial membrane and its distribution in the cytosol and plastids.

Although many organisms including plants are capable of synthesizing lipoic acid, the biosynthetic pathway is not fully described in any system (Parry, 1983). Very likely octanoic acid serves as a specific precursor of lipoic acid. One problem is the nature of the sulfur donor. The second problem associated with the conversion of octanoic acid into lipoic acid concerns the mechanism of the sulfur introduction process. Obviously a considerable work remains to be done in order to clarify lipoic acid biosynthesis in higher plant cells. We do believe that the synthesis of this important cofactor occurs in the mitochondrial matrix space because lipoic acid functions as the coenzyme in those mitochondrial multienzyme complexes that catalyze the oxidation of α-ketoacids and glycine. In support of this suggestion Wada et al. (1997) have demonstrated that pea leaf mitochondria contain not only acyl carrier protein (ACP), but all enzymes required de novo fatty acid synthesis. Their data suggested that a major part of the de novo synthesized fatty acids is used for biosynthesis of octanoic acid the putative precursor of lipoic acid. This last result could define the subcellular organization of lipoic acid biosynthesis in eukaryotes.

Highly specific lipoic protein ligases are required to attach lipoate to the appropriate lysine residues of the lipoate-dependent enzymes. This raises the question as to whether plant H-protein is lipoylated within the cytosol and then translocated into its final site of accumulation, or is targeted into mitochondria as apoprotein and subsequently lipoylated. Two major arguments support the mitochondrial lipoylation hypothesis. First, bovine mitochondria contain a lipoyl-protein ligase requiring lipoyl-AMP as a substrate (Fujiiwara et al., 1994). Second, Macherel et al. (1996) have constructed an expression system for mature pea H-protein in E. coli that allows production of large amounts of recombinant enzyme
as the apoform or the lipoylated form depending on the addition of lipoic acid to the growth medium. Very high rates of lipoylation of the H-apoprotein thus formed were measured in vivo and in vitro using intact bacteria or bacterial extract. Since the apoprotein was shown to have the same overall conformation as the native pea H-protein (Fig. 3) these results indicate that the lipoyl-protein ligase recognizes a three-dimensional structure in order to lipoylate the target lysine residue found at the extremity of the hairpin loop exactly as it is in the active protein. The lysine residue which become lipoylated is part of a conserved sequence motif V-K-A in which the two aminoacid residues V, A might be able to interact with lipoyl-protein ligase. Mutagenesis studies with the lipoyl domain from plant H-protein, however, indicate that these two aminoacid residues are not required for lipoylation (Macherel, Bourguignon and Douce, unpublished data). In conclusion the work of Macherel et al. (1996) strongly suggests that the attachment of lipoate to the appropriate lysine residue occurs once the H-protein is fully folded that is after its transport in the form of an uncoiled precursor through the mitochondrial inner membrane.

IV. Transfer of Reducing Equivalents from the Mitochondria and the Chloroplasts to the Peroxisomes

The conversion of hydroxypyruvate to glycerate in the peroxisomal matrix requires NADH as reductant. β-oxidation of fatty acids, the only pathway known in leaf peroxisomes to generate NADH (Beevers, 1979; Gerhardt, 1981), is too low in its activity to support the very high rates of hydroxypyruvate reduction required in an illuminated leaf. Peroxisomes are, therefore, dependent on the supply of reducing equivalents from outside. The question arises, whether the required NADH is delivered directly from the cytosol, or by redox shuttle, e.g. a malate-oxaloacetate shuttle.

In isolated peroxisomes, high rates of glycerate production from serine have been observed when NADH was added as reductant (Schmitt and Edwards, 1983; Reumann et al., 1994). A closer investigation of this reaction, however, revealed that under such conditions, due to lack of reductant (malate) in the peroxisomal matrix, hydroxypyruvate leaks out of the peroxisomes, and is reduced by hydroxypyruvate reductase present in the medium. Because of the very high activity of hydroxypyruvate reductase in the peroxisomes, the artificial release of a minor proportion of this enzyme to the medium is sufficient to catalyze the observed glycerate formation in the presence of added NADH. After correcting for this external reduction, the maximal velocity of glycerate production with external NADH as reductant was found to be one order of magnitude lower than with malate as reductant (Table 1). Moreover, the $K_M$ for NADH is approx. six times higher than the NADH concentration in the cytosol of a mesophyll cell, which is in the range of 1 μM (Heineke et al., 1991). With malate added as reductant, isolated peroxisomes produce glycerate at physiological rates. These results demonstrate that peroxisomes are supplied with reducing equivalents not by direct uptake of NADH but by indirect transfer via a malate-oxaloacetate shuttle.

Reducing equivalents can be delivered to the peroxisomes by malate-oxaloacetate shuttle from the mitochondria (Fig. 4), where an equimolar amount of the NADH required for hydroxypyruvate reduction is generated by the oxidation of glycine (Fig. 1). The NADH to NAD ratio in the mitochondria is more than 50 times higher than in the cytosol (Heineke et al., 1991) and in the peroxisomes (Raghavendra and Heldt, unpublished). NADH produced in the mitochondria can be re-oxidized rapidly by oxaloacetate (OAA), owing to the very high activity of malate dehydrogenase located in the matrix space. A very powerful phthalonate-sensitive oxaloacetate carrier has been characterized in all the plant mitochondria isolated so far (Douce and Neuburger, 1989). The rapid phthalonate-sensitive uptake of oxaloacetate is half-saturated at micromolar concentrations of oxaloacetate ($K_{OAA} = 5$ μM; $V_{max} = 2 \text{ μmol mg}^{-1} \text{ protein min}^{-1}$; the initial rates of oxaloacetate were measured using a rapid filtration technique within the first 150 ms and at 10°C (Neuburger and Douce, unpublished). This rapid malate-oxaloacetate transport shuttle, the equivalent

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<th>Reductant</th>
<th>$K_M$ [μM]</th>
<th>$V_{max}$ [μmol mg$^{-1}$ protein min$^{-1}$]</th>
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<tr>
<td>Malate</td>
<td>990</td>
<td>1.48</td>
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<td>NADH</td>
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of which is not found in mammalian mitochondria, appears to play an important role in the photorespiratory cycle, in catalyzing a substantial amount of reducing equivalents generated in the mitochondria during glycine oxidation to be transferred to the peroxisomal compartment for the reduction of $\beta$-hydroxypyruvate (Ebbighausen et al., 1985). This export of reducing equivalents from the mitochondria is readily visualized when small amounts of oxaloacetate are added to leaf mitochondria oxidizing glycine. At an extramitochondrial oxaloacetate concentration of 50 $\mu$M the influx of oxaloacetate is so severe that $NAD^+$-linked glycine dependent $O_2$ consumption comes to a stop because NADH is totally sequestered by soluble matrix malate dehydrogenase (the equilibrium of the malate dehydrogenase reaction lies far towards malate formation). Alleviation of respiratory inhibition subsequently occurs when the oxaloacetate is reduced. Interestingly, in the presence of oxaloacetate, the glycine decarboxylase reaction in isolated mitochondria is able to operate faster than when the NADH is reoxidized exclusively via respiratory complex I under state 3 conditions, i.e. during the coupling of electron transport to ATP production (Lilley et al., 1987). Because of the high activity of the mitochondrial malate-oxaloacetate shuttle (Manning and Heldt, 1993), the peroxisomal hydroxypyruvate reduction would act as a drain. The NADH generated from glycine oxidation would be totally consumed for the formation of malate and would be no longer available to support ATP synthesis by the respiratory chain. Mitochondrial ATP synthesis, however, is required during photosynthesis to supply energy to the cytosol of mesophyll cells (Krömer and Heldt, 1991b). In fact, mitochondria were found to deliver only about half of the reducing equivalents required for peroxisomal hydroxypyruvate reduction in a leaf. Chloroplasts provide the remaining portion (Krömer and Heldt, 1991; Hanning and Heldt, 1993a).

Also chloroplasts export reducing equivalents at a high rate by malate-oxaloacetate shuttle (Hatch et al., 1984) (Fig. 4). In this case the source is NADPH generated by non-cyclic electron transport, forming malate via NADP-malate dehydrogenase present in the stroma. Because of the high redox gradient between the NADPH/NADP system in the stroma and the NADH/NAD system in the cytosol, the transfer of redox equivalents from the stroma to the

\[ \text{Malate} \rightarrow \text{OAA} \rightarrow \text{Glycine decarboxylase} \rightarrow \text{OAA} \rightarrow \text{Malate} \rightarrow \text{Oxaloacetate} \rightarrow \text{NADPH} \rightarrow \text{NADH} \rightarrow \text{ATP} \]

\[ \text{NADPH} \rightarrow \text{NADH} \rightarrow \text{ATP} \rightarrow \text{NADH} \rightarrow \text{OAA} \rightarrow \text{Malate} \rightarrow \text{Oxaloacetate} \rightarrow \text{NADPH} \]

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\[ \text{NADPH} \rightarrow \text{NADH} \rightarrow \text{ATP} \rightarrow \text{NADH} \rightarrow \text{OAA} \rightarrow \text{Malate} \rightarrow \text{Oxaloacetate} \rightarrow \text{NADPH} \]

\[ \text{NADPH} \rightarrow \text{NADH} \rightarrow \text{ATP} \rightarrow \text{NADH} \rightarrow \text{OAA} \rightarrow \text{Malate} \rightarrow \text{Oxaloacetate} \rightarrow \text{NADPH} \]

\[ \text{NADPH} \rightarrow \text{NADH} \rightarrow \text{ATP} \rightarrow \text{NADH} \rightarrow \text{OAA} \rightarrow \text{Malate} \rightarrow \text{Oxaloacetate} \rightarrow \text{NADPH} \]

\[ \text{NADPH} \rightarrow \text{NADH} \rightarrow \text{ATP} \rightarrow \text{NADH} \rightarrow \text{OAA} \rightarrow \text{Malate} \rightarrow \text{Oxaloacetate} \rightarrow \text{NADPH} \]
cytosol has to be regulated. The site of regulation is the stromal NADP-malate dehydrogenase. The enzyme is activated by thioredoxin and therefore only active in the light (Rébeillé and Hatch, 1986a,b; Scheibe, 1987). In addition to this, increasing concentrations of NADP inhibit the reductive activation of the enzyme by thioredoxin. Thus a decrease in stromal NADP concentration, which corresponds to an increase of the chloroplastic NADPH to NADP ratio, switches on chloroplastic malate dehydrogenase. This allows the enzyme to function as a valve (Scheibe, 1990), through which excessive reducing equivalents can be exported from the chloroplast via a malate-oxaloacetate shuttle to the peroxisomes. In other words the NADPH to NADP ratio as well as the thioredoxin redox state may be critical in determining the level of NADP-malate dehydrogenase activity in vivo (Rébeillé and Hatch, 1986a,b).

V. The Compartmentation of Peroxisomal Metabolism

Glyoxylate and \( \text{H}_2\text{O}_2 \), formed as intermediates of the photorespiratory pathway are toxic to the plant cell. Photosynthesis of isolated chloroplasts is completely inhibited by the addition of low concentrations of glyoxylate and \( \text{H}_2\text{O}_2 \) (Flügge et al., 1980). Both substances react with SH-groups of thioredoxin-activated enzymes. The compartmentation of the conversion of glycolate to glycine in the peroxisomes allows the interconversion of these toxic products at the site of their formation so that they do not invade other cell compartments. Such compartmentation cannot be achieved by a membrane, since membranes are normally quite permeable for \( \text{H}_2\text{O}_2 \) and monocarboxylates (undissociated form) such as glyoxylate. In fact, the compartmentation of the reactions of the photorespiratory pathway in the peroxisomes is due to specific properties of the peroxisomal matrix.

When mitochondria or chloroplasts are exposed to an osmotic shock by suspending the organelles for a short time in distilled water, the boundary membranes of these organelles disrupt and the ‘soluble’ proteins (stroma, matrix), are released from the disrupted organelles, and metabolic compartmentation is abolished. When peroxisomes, however, are suspended for 10 min at 20 °C in distilled water, the boundary membrane is ruptured, but the matrix proteins remain aggregated in the form of particles in the size of peroxisomes, and the compartmentation of peroxisomal reactions is maintained (Heupel et al., 1991). Intermediates of peroxisomal metabolism, i.e. glyoxylate, \( \text{H}_2\text{O}_2 \) and hydroxypyruvate, are not released from these particles during glycolate oxidation. This finding allows the conclusion that the compartmentation of peroxisomal metabolism is not caused by the boundary membrane. Apparently the enzymes of the photorespiratory pathway are arranged in the peroxisomal matrix in the form of a multi-enzyme complex, by which the product of a reaction binds immediately to the enzyme of the following reaction and is therefore not released, in a process referred to as metabolite channeling (Heupel and Heldt, 1994).

As even in life nothing is perfect, one might assume that in spite of metabolite channeling, it may happen that a minor portion of glyoxylate or hydroxypyruvate escapes from the peroxisomes. For such a case there are rescue enzymes present in the cytosol which use NADPH to convert glyoxylate to glycolate (NADPH-glyoxylate dehydrogenase) and hydroxypyruvate into glycerate (Givan and Kleczkowski, 1992).

VI. Transfer of Metabolites Across the Peroxisomal Boundary Membrane

In the photorespiratory pathway eight different metabolites have to cross the peroxisomal membrane. Previous attempts by several research groups to identify specific metabolite translocators in the peroxisomal membrane similar to those of mitochondria and chloroplasts have been unsuccessful. Instead, using the planar lipid bilayer technique, it was found that leaf peroxisomes as well as glyoxysomes contain a pore forming protein (Reumann et al., 1995, 1996, 1997). Compared with the porin from the outer membrane of mitochondria and chloroplasts, the peroxisomal pore has a comparatively small opening, since its single channel conductance (350 pS in 1 M KCl) is much lower than those formed by the porins of mitochondria and chloroplasts (Table 2). As a special feature, the peroxisomal pore contains an anion binding site with a high affinity for short chain dicarboxylates, such as malate, oxaloacetate and 2-oxoglutarate (Reumann et al., 1998). Glycolate and glycerate were also found to permeate the pore (Reumann et al., 1998).
Apparently, the peroxisomal channel is not a wide, water-filled channel like the channels of the mitochondrial and the chloroplast porins but has specific properties comparable to specific and inducible porins, which have been characterized in some gram-negative bacteria. These findings indicate that the porin-like channel which is slightly anion selective in accordance with its physiological function, represents the major permeability pathway of the peroxisomal membrane.

VII. Concluding Remarks

The oxygenation of RuBP by Rubisco is looked upon as a side reaction, which nature was unable to avoid (Andrews and Lorimer, 1978). The reason is probably that early evolution of Rubisco occurred at a time when there was no oxygen in the atmosphere. When, more than a billion years later, due to photosynthesis, oxygen appeared in the atmosphere in higher concentrations, the complexity of Rubisco protein probably made it too difficult to change the catalytic center to eliminate oxygenase activity linked to glycolate-2-P production. Rubisco inevitably initiates inefficiencies of carbon assimilation (Osmond and Grace, 1995). Unlike respiration producing ATP, photorespiration which is a consequence of the oxygenation of RuBP by Rubisco consumes ATP. The recycling of glycolate-2-P into glycerate-3-P via the C₃ cycle and then further to RuBP is not only a very costly reaction, consuming one third or more of the total energy requirement of CO₂ fixation, it also requires a large machinery, consisting of more than 15 enzymes and translocators, distributed over three different organelles, i.e. the chloroplast, peroxisome and mitochondrion (fig. 1). Photorespiratory metabolism is able to prevent the dramatic accumulation of glycolate as well as the formation of the excited triplet state of chlorophyll and excess reactive O₂ species (superoxide radicals and singlet oxygen) which would be damaging to the chloroplast membranes (photoinhibition) (Osmond and Grace, 1995). In other words photorespiration, in concert with other reactions (Halliwell-Asada cycle), potentially mitigates chronic photoinhibition. Once glycolate-2-P is formed, the photorespiratory cycle works forward to convert all the carbon diverted out of the C₃ cycle back to photosynthesis as rapidly as possible (Lorimer and Andrew, 1981). Indeed several reactions occurring in chloroplasts and peroxisomes strongly favor product formation: that is the forward motion of the photorespiratory cycle. In addition, the rapid utilization of NADH, and the immediate utilization of NH₄⁺ (via glutamate synthase and glutamine synthetase operating in a concerted manner) and CO₂ (via Rubisco) during the course of mitochondrial glycine oxidation continuously shift the equilibrium of both reactions catalyzed by glycine decarboxylase and SHMT towards serine production even though the reaction are readily reversible in vitro. Likewise, serine is rapidly removed from the mitochondria in order to allow the continuous production of serine by SHMT, the equilibrium of which would otherwise be unfavorable (Besson et al., 1993). Obviously the only control step in the photorespiratory cycle is at the level of competition between O₂ and CO₂ for binding to Rubisco.

It appears certain that the introduction of a genetic approach complements the more classical methods used in the study and regulation of photorespiration. Two major problems remaining for the future are: a) despite few impressive advances (Weber et al., 1996), it is fair to say that we still do not have a clear idea as to how any of these transporters directly involved in photorespiratory cycle function at the molecular level to translocate substrates across the chloroplastic and mitochondrial membranes. In addition, the purification and functional reconstitution as well as the completion of detailed kinetic analyses of most of

<table>
<thead>
<tr>
<th>Porin from</th>
<th>Single channel conductance [ns]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxisome (spinach)</td>
<td>0.35</td>
<td>Reumann et al., 1995</td>
</tr>
<tr>
<td>Glyoxysome (castor bean)</td>
<td>0.34</td>
<td>Reumann et al., 1997</td>
</tr>
<tr>
<td>Mitochondrion (pea)</td>
<td>3.7</td>
<td>Schmid et al., 1993</td>
</tr>
<tr>
<td>Chloroplast (spinach)</td>
<td>7.8</td>
<td>Flügge and Benz, 1984</td>
</tr>
</tbody>
</table>
these transporters should be undertaken. Such information will prove valuable to our understanding of the full spectrum of molecular mechanisms that chloroplasts and mitochondria utilize to catalyze the rapid metabolite flows across the inner membranes during the course of photorespiration.

Finally, an intriguing question is how the coordinated control of a multitude of genes in a precise spatial and temporal program, can lead to the development of this exquisite photorespiratory cycle.

Acknowledgments

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Summary

During photosynthesis, energy from solar radiation is used to convert atmospheric CO₂ into intermediates that are used within, as well as outside, the chloroplast for a series of metabolic pathways. These intermediates have to be exchanged between the chloroplast and the cytosol. The envelope membrane of chloroplasts contains various specific translocators that are involved in these transport processes. The basic properties of several of these translocators have been extensively studied in the past. The elucidation of the molecular structure of some of these translocators during the last years opened the way to study the function of particular translocators in more detail. This chapter focuses on the progress achieved in this field with emphasis on the characterization of different classes of phosphate translocators and on translocators that are specific for dicarboxylates, adenylates and hexoses.
I. Introduction

Communication between chloroplasts and the surrounding cytosol occurs via the plastid envelope membrane. The outer envelope possesses pore forming proteins (porins) that allow the nonspecific diffusion of hydrophilic solutes. The inner envelope membrane contains various metabolite transporters that mediate the exchange of metabolites between both compartments (Flügge and Heldt, 1991). The carbon fixed during the day can be exported from the chloroplasts into the cytosol for the synthesis of sucrose which is subsequently allocated to heterotrophic organs of the plant such as roots, seeds, fruits or tubers. When the rate of sucrose biosynthesis and export falls below that of CO₂ assimilation, the fixed carbon is retained in the chloroplasts and directed into the biosynthesis of assimilatory starch. Remobilization of starch during the following dark period and export of the starch breakdown products ensures a continuous supply of photosynthates to heterotrophic tissues in the dark. Two chloroplast translocators are involved in these processes: (1) The triose phosphate/phosphate translocator (TPT), a member of the phosphate translocator family, that exports the fixed carbon (in the form of trioseP and 3-PGA) from the chloroplast into the cytosol, where it is converted into other substances, e.g. sucrose and amino acids (see Fig. 1). The inorganic phosphate released during these biosynthetic processes is reimported into the chloroplast via the TPT for the formation of ATP catalyzed by the thylakoid ATP synthase.

Sucrose and amino acids are the main products for the supply of heterotrophic tissues with photosynthates. They are actively loaded into the sieve element/companion cell complex by specific H+/symporters. The isolation and elucidation of the molecular structures of transporters for sucrose and various amino acids have been recently achieved using a yeast complementation system (for review see Frommer and Ninnemann, 1995; Ward et al., 1997; Fischer et al., 1998).

Transport mediated by the TPT was first investigated in intact chloroplasts (Fliege et al., 1978) and later, after purification and cloning of the corresponding cDNA, in artificial membranes containing the recombinant expressed protein (see below). In its functional form, the TPT is a dimer composed of two identical subunits (Flügge, 1985; Wagner et al., 1989). As substrates, the TPT accepts either inorganic phosphate or a phosphate molecule attached to the end of a three-carbon chain, such as trioseP or 3-PGA. C3-compounds with the phosphate molecule at C-atom 2 (phosphoenolpyruvate, 2-PGA) are only poorly transported (see below). Under physiological conditions, the substrates are transported via a strict 1:1 exchange. Transport proceeds via a ping-pong type of reaction mechanism, i.e. the first substrate has to be transported across the membrane and then leave the transport site before the second substrate can be bound and transported to the opposite direction (Flügge, 1992). In intact chloroplasts, unidirectional transport of phosphate can be observed but with a Vₘₐₓ that is two to three orders of magnitude lower as compared to the antiport mode (Fliege et al., 1978; Neuhaus and Maass, 1996). Using the reconstituted system in which the concentrations of phosphate in both the internal and the external compartments are accessible to experimental variations, it could be demonstrated that the transport activity of the reconstituted TPT can reach values that exceed those measured for an antiport mode by at least one order of magnitude. It is suggested that transport under these conditions proceeds by a mechanism different from the antiport mode, probably by a (channel-like) uniport mechanism. Evidence for ion channel

Abbreviations: DiT1 – 2-oxoglutarate/malate translocator; DiT2– glutamate/malate translocator; GS – glutamine synthetase; OEP – outer envelope protein; PEP – phosphoenolpyruvate; 2-PGA – 2-phosphoglycerate; 3-PGA – 3-phosphoglycerate; PPT – phosphoenolpyruvate/phosphate translocator; TPT – triose phosphate/3-phosphoglycerate/phosphate translocator; TrioseP – triose phosphates; VDAC – voltage-dependent anion channel translocator (TPT) mediates the export of the fixed carbon (in the form of trioseP and 3-PGA) from the chloroplast into the cytosol, where it is converted into other substances, e.g. sucrose and amino acids (see Fig. 1). The inorganic phosphate released during these biosynthetic processes is reimported into the chloroplast via the TPT for the formation of ATP catalyzed by the thylakoid ATP synthase.

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properties of the TPT is provided, (i) by a decrease of the activation energy for phosphate transport from 46 kJ/mol (antiport mode) to 18 kJ/mol (uniport mode), a value that is in the range observed for ion channels and, (ii) by measuring the TPT-mediated unidirectional transport by the patch-clamp technique (Schwarz et al., 1994). It can be concluded from these electrophysiological experiments that the PT can behave as a voltage-dependent ion channel, preferentially permeable to anions, as well as an antiporter. Different classes of transporters might share common structural motifs and may have arisen from a common ancestor. A small structural change within the translocation pathway might then allow transport via an ion channel mode or might result in strong coupling of substrate binding with conformational changes as observed for transporters operating in the antiport mode.

The spinach TPT was the first plant membrane transport system for which the primary sequence could be determined (Flügge et al., 1989). Meanwhile, TPT-sequences from various plants are available, e.g., those from Arabidopsis, pea, potato, maize, Flaveria and tobacco that all have a high similarity to each other (Knight and Gray, 1994; Fischer et al., 1994a, 1997). All TPTs are nuclear-encoded and possess N-terminal transit peptides (about 80 amino acid residues) that direct the adjacent protein to the chloroplasts (Brink et al., 1995; Knight and Gray, 1995). Import of the translocator into chloroplasts is driven by ATP and depends on the translocation machinery of the envelope membrane. The mature part of these transporters consists of about 330 amino acid residues per monomer, is highly hydrophobic, and contains information (envelope insertion signals) for the integration of the protein within the inner envelope membrane. It is composed of five to seven hydrophobic segments in an α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic loops. The TPT thus belongs to the group of translocators with a 6 + 6 helix folding pattern, as is the case for mitochondrial carrier proteins (Maloney, 1990). Based on a tentative model for the arrangement of the TPT in the membrane, it is probable that all twelve α-helices take part in forming a hydrophilic translocation channel through which the substrates are transported across the membrane. According to the proposed three-dimensional structural model of the TPT (Wallmeier et al., 1992), an asymmetry in the structure of the substrate binding sites on either membrane side is suggested. This structural asymmetry was verified experimentally by demonstrating that only the cytosolic side of the translocator is accessible to specific inhibitors, namely pyridoxal 5′-phosphate and 4,4′-diisothiocyanostilbene-2,2′-disulfonate (Flügge, 1992). Bisubstrate initial velocity studies of the reconstituted translocator further showed that the TPT possesses a higher affinity (about five-fold) toward its substrates on the cytosolic side.
compared to the stromal side.

The availability of the TPT-cDNA also opened the way to produce the corresponding protein in heterologous expression systems. Since prokaryotic systems failed to express the hydrophobic translocator protein, yeast cells (*Schizosaccharomyces pombe*) were used. It could be shown that the TPT was produced in yeast cells in a functional state representing about 1% of the total protein (Loddenkötter et al., 1993). To facilitate the isolation of the TPT from the cells, a recombinant protein was engineered containing a C-terminal tag of six consecutive histidine residues (His₆-tag). This was initially achieved by overlapping PCR; however, in the meanwhile, various commercially available vectors can be used for introducing different kinds of tags. Despite the low level of expression, the TPT-His₆ protein could be purified to apparent homogeneity by a single chromatography step on metal-affinity columns. It could be demonstrated that the recombinant protein possessed transport characteristics almost identical to those of the authentic chloroplast protein (Loddenkötter et al., 1993).

2. Physiological Role of the TPT

The TPT is an important link between metabolism in the chloroplast and the cytosol. It can be calculated that only about 10% of the total transport activity of the TPT can be used for (productive) net trioseP export in order to provide the carbon skeleton for further biosynthetic processes. Since both the cytosol and the stroma contain trioseP, 3-PGA and inorganic phosphate competing for transport in either direction, it appears feasible that most of the TPT activity is used for catalyzing (non-productive) homologous exchanges (Flügge, 1987).

From subcellular metabolite concentrations in intact spinach leaves, it has been proposed that the TPT can exert a kinetic limitation during sucrose biosynthesis in vivo (Gerhardt et al., 1987). We have assessed the role of the TPT on photosynthetic metabolism by means of antisense repression of its corresponding mRNA. Transgenic potato (*Solanum tuberosum* L.) plants were created that showed a dramatically reduced expression of the TPT at the RNA level (Riesmeier et al., 1993). In all transgenic plants analyzed, the maximum reduction of both the amount and the activity of the TPT was about 25–30%. Under ambient CO₂ and light conditions, there was no significant effect on photosynthetic rates (Table 1), growth, and tuber development. However, even under ambient conditions, the reduction of the TPT activity resulted in a marked perturbation of leaf metabolism. Most remarkably, the content of 3-PGA was greatly increased compared to the corresponding value in wild-type plants and this increase was found to be almost entirely due to an increase of the stromal 3-PGA content with the cytosolic values remaining almost unchanged. Also, the sum of organic phosphates (3-PGA plus sugar phosphates) was about twice as large in the transformants as in the wild-type plants. This should result in a large decrease of the stromal content of inorganic phosphate since the TPT mediates a strict counter-exchange of substrates. Thus, reduction of the TPT activity in vivo resembles the situation of chloroplasts performing photosynthesis under phosphate limitation (Heldt et al., 1977): the increase in 3-PGA (and the 3-PGA/phosphate ratio) results in a large increase of starch synthesis due to the allosteric activation of the ADP-glucose pyrophosphorylase (AGPase) (Preiss and Levi, 1980). Indeed, both at the end of the day and end of the night the starch content

<table>
<thead>
<tr>
<th>Plants</th>
<th>Wild-type</th>
<th>TPT 1</th>
<th>TPT 7</th>
<th>TPT 39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake of [³²P] phosphate into chloroplasts (% of Vₘₜₐₓ)</td>
<td>= 100</td>
<td>72</td>
<td>69</td>
<td>83</td>
</tr>
<tr>
<td>Photosynthetic rate, ambient conditions (mmol O₂ per m² per h)</td>
<td>17.5</td>
<td>21.9</td>
<td>14.3</td>
<td>19.8</td>
</tr>
<tr>
<td>Starch (end of the day) (μatom C per mg Chl.)</td>
<td>761</td>
<td>1310</td>
<td>2101</td>
<td>1336</td>
</tr>
<tr>
<td>Starch (end of the night) (μatom C per mg Chl.)</td>
<td>330</td>
<td>654</td>
<td>1291</td>
<td>505</td>
</tr>
</tbody>
</table>
in transformants was much greater than in wild-type plants (Table 1). It appears that due to the restriction of the TPT, the daily assimilated carbon is mainly maintained within the plastids in which it is directed into the accumulation of starch (Table 2). Since there was no obvious effect on plant growth, the transformants were obviously able to efficiently compensate for their deficiency in TPT activity. A detailed analysis of the transgenic potato plants indeed showed that these plants mobilized and exported the major part of the daily accumulated carbon during the following night period (Heineke et al., 1994). By contrast, wild-type plants generally export the major part of the fixed carbon during ongoing photosynthesis (Table 2).

The question arises how the mobilization and export of photoassimilates is achieved in the antisense TPT plants during the night. Mobilization of starch and export of the resulting breakdown products can, in principle, either be performed via the TPT (after phosphorylolytic starch breakdown resulting in trioseP) or via a hexose translocator (after amylolytic starch breakdown resulting in hexoses, see Fig. 1). Due to the reduced activity of the TPT, the mobilized carbon cannot be exported (at least not at high rates) from the chloroplasts as C3-compounds that are end-products from phosphorylolytic starch breakdown. Obviously, the transformants follow the hydrolytic starch breakdown pathway and preferentially export the mobilized carbon as hexoses via the hexose translocator. This is in line with the observation that transgenic antisense TPT plants from tobacco showed increased rates of amylolytic starch mobilization which process is accompanied by a higher transport capacity for glucose across the envelope membrane (Häusler et al., 1998). It can be concluded that plants can efficiently compensate for their deficiency in TPT activity provided that a carbon sink (i.e., starch) can be generated during photosynthesis which can be mobilized during the following night period. Interestingly, transgenic potato plants with a reduced ability to synthesize assimilatory starch (e.g., by anti-sense repression of the AGPase) also show no effect on growth and productivity. Export of the daily fixed carbon via the TPT is obviously so efficient in these plants that heterotrophic tissues can be supplied sufficiently with reduced carbon. However, if both starch formation and the activity of the TPT are reduced, the corresponding transformants show a dramatic phenotype (Hattenbach et al., 1997). On the one hand, the transformants are not able to export sufficient amounts of fixed carbon during the day (due to antisense repression of the TPT) and on the other hand, they do not have a carbon store which they could use during the dark period (due to antisense repression of the AGPase).

**B. The Phosphoenolpyruvate/Phosphate Translocator**

1. **Structure and Function**

Another type of phosphate translocator has been described recently (Fischer et al., 1997). This transporter, named PPT (phosphoenolpyruvate/phosphate translocator) was initially identified in non-green tissues, but it is also present in photosynthetic tissues. As in the case of the TPT, the PPTs are nuclear-encoded and consist of a transit peptide (about 80 amino acid residues) and a mature part (about 320 amino acid residues). Sequence comparisons between the TPT and the PPTs from different plants and plant tissues showed that the PPTs represent a second class of phosphate translocators (Table 3). Within each group of transporters, the mature proteins are highly homologous to each other. In contrast, the

<table>
<thead>
<tr>
<th>Plants</th>
<th>Wild-type</th>
<th>TPT 39</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Amount of assimilate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) assimilated during the day</td>
<td>1085</td>
<td>975 $\mu$atom C mg Chl.$^{-1}$</td>
</tr>
<tr>
<td>b) accumulated during the day</td>
<td>466 (43%)</td>
<td>866 (89%) $\mu$atom C mg Chl.$^{-1}$</td>
</tr>
<tr>
<td>2. Rate of assimilate export</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) during the day</td>
<td>52 (57%)</td>
<td>9 (11%) $\mu$atom C mg Chl.$^{-1}$ h.$^{-1}$</td>
</tr>
<tr>
<td>b) during the night</td>
<td>39 (43%)</td>
<td>72 (89%) $\mu$atom C mg Chl.$^{-1}$ h.$^{-1}$</td>
</tr>
</tbody>
</table>
homologies between members of the TPT family and the PPT family, respectively, are only about 35%.

To study the transport characteristics of the cloned PPT proteins, the cDNA from the cauliflower PPT3 clone was fused to a DNA fragment encoding a tag of six consecutive histidine residues (His6-tag) and was then subcloned into a yeast expression vector. As outlined above, this procedure enables the one-step purification of the produced transporter to apparent homogeneity by metal-affinity chromatography for the subsequent analysis of the transport characteristics by reconstitution experiments (Loddenkötter et al., 1993).

Figure 2 shows the substrate specificities of the purified PPT and the TPT, both purified from yeast cells, as determined by measuring transport into proteoliposomes that had been preloaded with the indicated counter-substrates. Both classes of phosphate translocators greatly differ in their transport characteristics. The PPT protein transports inorganic phosphate preferentially in exchange with PEP. This is in sharp contrast to the transport characteristics of the TPT. This transporter accepts either inorganic phosphate or C3-compounds that are phosphorylated at C-atom 3 i.e., triose phosphates and 3-phosphoglycerate (see above). These substrates, however, are only poorly transported by the PPT protein. Both types of transporters strictly rely on the presence of an exchangeable substrate within the vesicles (i.e., they function as antiport systems) and do not transport hexose phosphates like glucose 6-phosphate at all.

### 2. Physiological Role of the PPT

Do plastids rely on externally produced PEP or can PEP be generated inside the organelles? Work from several laboratories has shown that the conversion of hexose phosphates and/or triose phosphates via glycolysis cannot proceed further than to 3-phosphoglycerate due to the absence (or low activities) of phosphoglycerate mutase and/or enolase in most plastids (Stitt and ap Rees, 1979; Schulze-Siebert et al., 1984; Journet and Douce, 1985; Van der Straeten et al., 1991; Miernyk and Dennis, 1992; Borchert et al., 1993). These plastids depend therefore on the provision of externally produced PEP. This fact has been overlooked to date, or it has been assumed that the transport of PEP into chloroplasts is facilitated by the TPT. This, however, appears unlikely because PEP is only poorly accepted as a substrate by the TPT (Fig. 2) and has, under physiological conditions, to compete with inorganic phosphate, trioseP, and 3-phosphoglycerate for binding to the TPT. Hence, the presence of a PPT that bypasses the TPT is the most likely alternative for an efficient provision of the chloroplasts with PEP.

Several processes inside the plastids rely on PEP (see Fig. 3). This compound is an immediate substrate for the shikimate pathway which leads to the biosynthesis of aromatic amino acids and to a large number of secondary compounds that are important in plant defense mechanisms and stress responses (for review see Herrmann, 1995). It has also been shown that the plastid-located fatty acid biosynthesis can be driven by exogenous pyruvate (Liedvogel and Bäuerle, 1986). Plastidic acetyl-CoA can subsequently be formed by the action of either acetyl-CoA synthetase or via the plastidic pyruvate dehydrogenase complex. In those plastids missing a complete pathway from 3-phosphoglycerate to pyruvate, PEP could be imported into the organelles via the PPT and pyruvate kinase could subsequently convert PEP into pyruvate.

---

**Table 3. Homologies (in %) between the chloroplast triose phosphate/phosphate translocators (TPT) and the phosphoenolpyruvate/phosphate translocators (PPT) from different plants.**

<table>
<thead>
<tr>
<th>TPTs from</th>
<th>Spinach</th>
<th>Cauliflower</th>
<th>Maize</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach TPT</td>
<td>(100)</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>Cauliflower TPT</td>
<td>(100)</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>Maize TPT</td>
<td>(100)</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>Cauliflower PPT3</td>
<td>(100)</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>Arabidopsis PPT12</td>
<td>(100)</td>
<td>93</td>
<td>77</td>
</tr>
<tr>
<td>Tobacco PPT10</td>
<td>(100)</td>
<td>80</td>
<td>73</td>
</tr>
<tr>
<td>Maize PPT1</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6 Chloroplast Transport

Fig. 2. Substrate specificities of the TPT and the PPT. The cauliflower PPT3-His₆ protein and the spinach TPT-His₆ protein were expressed in Schizosaccharomyces pombe cells and purified from these cells by metal affinity chromatography (Loddenkötter et al., 1993). The recombinant proteins were reconstituted into liposomes that had been preloaded with 25 mM substrates as indicated. [³²P]phosphate transport activity was measured as described by Fischer et al. (1997) and is given as a percentage of the activity measured for proteoliposomes preloaded with inorganic phosphate. The 100% exchange activities (µmol mg⁻¹ protein x min⁻¹) were 1.5 (PPT; dark gray, left), and 0.85 (TPT; light grey, right). 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; TrioseP, dihydroxyacetone phosphate; Glu6-P, glucose 6-phosphate.

Fig. 3. Proposed function of the PPT protein in photosynthetically active tissues. The combined action of the TPT and the PPT results in the supply of the organelle with PEP generated from photosynthetically fixed carbon. 3-PGA, 3-phosphoglycerate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; Ery4P, erythrose 4-phosphate; Fru6P, fructose 6-phosphate; RuBP, ribulose 1,5-bisphosphate; TrioseP, triose phosphates.
Figure 3 outlines the proposed physiological function of the PPT in chloroplasts. The fixed carbon is exported from the chloroplast by the TPT and partially converted into PEP in the cytosol. The PPT can provide the chloroplast stroma with PEP for the shikimate pathway. In addition, PEP can be converted into pyruvate as a precursor for fatty acid biosynthesis, concomitantly providing the plastids with ATP. The latter issue is probably important in chloroplasts during night or in non-photosynthetic tissues. Because inorganic phosphate is used as a counter-substrate by both the TPT and the PPT, the combined action of both translocators would result in an exchange of triose phosphate with PEP without net phosphate transport.

With the identification of a phosphate translocator specific for PEP, it is proposed that plastids contain a set of phosphate translocators with different structures but overlapping substrate specificities. Chloroplasts, or at least a subtype of chloroplasts, contain members of both the TPT and the PPT family. The TPT is present only in photosynthetic tissues (Schulz et al., 1993, Flügge, 1995), whereas PPT-specific transcripts can be detected in both leaves and non-green tissues although they are more abundant in non-green tissues (Fischer et al., 1997). The presence of different phosphate translocators with different substrate specificities would allow the uptake of any particular phosphorylated substrates even in the presence of high concentrations of other phosphorylated metabolites, which would otherwise compete for the binding site of a single phosphate translocator.

C. The Hexose Phosphate/Phosphate Translocator

It has been shown that chloroplasts from guard-cells contain a phosphate translocator that also transports glucose 6-phosphate (Overlach et al., 1993). Stomatal opening is associated with the production of malate as a counterion for potassium. Malate can derive from starch mobilization, the products of which are exported from the chloroplasts and used for malate formation. For the biosynthesis of starch, guard-cells have to import reduced carbon. It has been proposed that trioseP and/or 3-PGA, imported via the chloroplast phosphate translocator, can be used for the formation of hexose phosphates (Preiss et al., 1985). However, guard-cells are devoid of fructose 1,6-bisphosphatase activity (Hedrich et al., 1985), the key enzyme for the conversion of trioseP into hexose phosphates, and therefore, rely on the provision of hexose phosphates for starch biosynthesis. In this respect, guard-cell chloroplasts behave like non-green plastids that also lack fructose 1,6-bisphosphatase activity (Entwistle and ap Rees, 1988) and have to import hexose phosphates for starch biosynthesis. Both organelles obviously contain a phosphate translocator that can import hexose phosphates in exchange with inorganic phosphate.

A hexose phosphate/phosphate transport activity can also be detected in spinach chloroplasts after feeding of detached leaves with glucose (Quick et al., 1995). The chloroplasts of these leaves are photosynthetically active but contain large quantities of starch. As is the case for heterotrophic plastids in sink tissues, the precursor for starch biosynthesis is imported into the chloroplasts in the form of glucose 6-phosphate by a novel hexose phosphate/phosphate translocator. Glucose-feeding has obviously induced a switch in the function of the chloroplasts from carbon-exporting source organelles to carbon-importing sink-organelles. These experiments show that the physiological state of a tissue can greatly alter its biochemical properties and demonstrate the high flexibility of plant metabolism.

We have recently identified a translocator that mediates the transport of glucose 6-phosphate in exchange with either inorganic phosphate or triose phosphates (glucose 6-phosphate/phosphate translocator, GPT) (Kammerer et al., 1998). Glucose 6-phosphate imported into the plastids via the GPT can thus be used either for starch biosynthesis, during which process inorganic phosphate is released, or as a substrate for the oxidative pentose phosphate pathway, yielding triose phosphates. The GPT shares about 38% identical amino acids with members of the TPT and PPT families and thus represents a third group of plastidic phosphate antiporters. The GPT gene is predominantly expressed in heterotrophic tissues but is most probably also responsible for the above described hexose phosphate transport activities in chloroplasts.

III. Transport of Glucose

The amylolytic degradation of starch at night results in the formation of glucose that can be exported from the chloroplasts by a glucose translocator and...
converted to sucrose in the cytosol (see Fig. 1). As mentioned above, transgenic anti-sense TPT potato plants export carbon during the night preferentially via this translocator to compensate for their deficiency in TPT activity (Heineke et al., 1994). Evidence that the preferred route for exporting carbon from chloroplasts proceeds via the glucose translocator comes from studies on a high starch Arabidopsis mutant (starch-excess mutant TC265, Caspar et al., 1991). This mutant is able to degrade starch but obviously unable to export the products of starch degradation. These are therefore redirected into the synthesis of starch leading to the observed starch-excess phenotype. The chloroplasts possess a functional TPT but obviously lack a functional chloroplast glucose translocator for carbon export (Trethewey and ap Rees, 1994). Little is known about this translocator that had first been described in the late seventies (Schäfer et al., 1977). The availability of starch-excess mutants will presumably open the way to isolate the corresponding gene, e.g., by map-based cloning.

### IV. Dicarboxylate Translocators

The photosynthetically fixed carbon that is exported from the chloroplasts via the TPT as trioseP and 3-PGA can also be used for the formation of carbon skeletons (2-oxoglutarate) via processes occurring in the cytosol and in the mitochondria. 2-oxoglutarate can then be imported into the chloroplasts for the fixation of ammonia that derives from nitrate reduction or photorespiration. Fixation of ammonia within the chloroplast is achieved via the stromal glutamine synthetase (GS2)/glutamate synthase cycle. Glutamate synthesized during this cycle is the key compound of nitrogen metabolism in plants. It can be subsequently exported into the cytosol (Fig. 4).

Two different dicarboxylate antiport systems are involved in this process. Both translocators mediate an exchange with malate: (i) a 2-oxoglutarate/malate translocator (DiT1) that transports 2-oxoglutarate into the chloroplasts in exchange with malate and that does not transport glutamate or aspartate and (ii) a glutamate/malate translocator (DiT2) exporting glutamate in exchange with cytosolic malate. DiT2 can be considered as a general dicarboxylate

![Diagram of chloroplast transport](image)

**Fig. 4.** Two plastidic dicarboxylate translocators are involved in ammonia assimilation. DiT1, 2-oxoglutarate/malate translocator; DiT2, glutamate/malate translocator; GS/GOGAT, glutamine synthetase/glutamate synthase; P₃, inorganic phosphate; RuBP, ribulose 1,5-bisphosphate; TrioseP, triose phosphates.
translocator. Because both translocators use malate as the substrate for counter-exchange, the resulting 2-oxoglutarate/glutamate transport proceeds without net malate transport (Woo et al., 1987; Flügge et al., 1988). Glutamate and other amino acids can then be further loaded into the sieve tubes via specific amino acid transporters.

Mutants of *Arabidopsis* deficient in dicarboxylate transport activities are not viable under photorespiratory conditions (Somerville and Somerville, 1985). The defect is probably caused by the general dicarboxylate translocator (DiT1). However, attempts to clone the corresponding gene have failed so far.

The identification of the 2-oxoglutarate/malate translocator (DiT1) as a 45 kDa component of the inner envelope membrane has recently been described (Menzlaff and Flügge, 1993). Subsequently, a cDNA clone was obtained that codes for the precursor protein with a relative molecular mass of 60 kDa (Weber et al., 1995). Interestingly, there is no detectable homology to any other known protein, not even to the mitochondrial 2-oxoglutarate/malate carrier with transport characteristics similar to DiT1 (Runswick et al., 1990).

The mature part of DiT1 contains 13-14 hydrophobic segments; 12 of them are long enough to span the membrane as transmembrane α-helices. These helices are obviously arranged in such a way that two clusters of six helices each are separated by an intervening hydrophilic loop. This transmembrane topology with an 12-helix motif resembles that of other plasmamembrane transporters from prokaryotes and eukaryotes that presumably all function as monomers. The recently identified plastidic ADP/ATP translocator also belongs to this type of transporter (see below). All other organellar transporters, including those from mitochondria, belong to the second group of transporters having 5-7 transmembrane helices like the chloroplast phosphate translocators. These translocators probably function as homodimers with a ‘6 + 6’ transmembrane helix pattern. Thus, the inner envelope membrane possesses two different types of transporters which may all have, in their functional state, a comparable number of membrane-spanning segments.

To verify the identity of the cloned gene, the full-length cDNA sequence was expressed in the fission yeast *Schizosaccharomyces pombe*. Yeast transformants harboring the DNA coding for DiT1 indeed displayed reconstituted malate transport activities at rates exceeding those of the endogenous transporters by at least two orders of magnitude. It could be further shown that substrate specificities of the recombinant protein were almost identical to the translocator purified from envelope membranes (Weber et al., 1995). The activity of the recombinant protein is thus in accordance with its proposed function as a supplier of the chloroplasts with 2-oxoglutarate for ammonia assimilation.

It has been shown that chloroplasts contain, in addition to DiT1 and DiT2, a specific translocator for glutamine with glutamate as the preferential countersubstrate. Glutamine is not transported by DiT1, and by DiT2 only at low rates. It has been proposed that the glutamine translocator is involved in the refixation of photorespiratory ammonia by mediating uptake of glutamine formed by the cytosolic isofrom of glutamine synthetase (GS1) (Yu and Woo, 1988). However, it has been shown that the presence of GS1 is exclusively restricted to the cytoplasm of phloem companion cells whereas the chloroplast-located GS2 is localized in mesophyll cells in which it is involved in nitrogen assimilation (Edwards et al., 1990; Carvalho et al., 1992). Since the phloem companion cells do not contain photosynthetically active chloroplasts, uptake of GS 1-produced glutamine into the plastids is not possible. Although the location of the glutamine/glutamate translocator has not yet been determined, it is suggested that this translocator resides in the mesophyll. In these cells, glutamine, produced by the stromal GS2, could be exported from the chloroplasts in exchange for cytosolic glutamate. This would allow a supply of assimilated nitrogen to the cell.

Chloroplasts are also able to transport oxaloacetate (OAA). Import of OAA into chloroplasts can be closely linked to an export of malate. This malate/OAA shuttle plays an important role in the transfer of reducing equivalents from the chloroplasts into the cytosol (Heineke et al., 1991). In principle, OAA can be transported by DiT1 and DiT2. However, this appears unlikely because the concentrations of OAA in both the cytosol and the stroma are at least one order of magnitude lower than that of malate and other dicarboxylates with which substrates OAA has countertransported.measurement of the uptake of OAA into chloroplasts of *C. vulgaris* revealed the presence of a specific translocator with a high affinity for OAA. This translocator is able to transport OAA even in the presence of a large excess of other dicarboxylates...
(Hatch et al., 1984). Imported OAA can also be used for transamination to aspartate which is, in turn, the precursor for the biosynthesis of various amino acids (Bryan, 1990). The molecular structure of the OAA translocator is yet unknown.

C3-chloroplasts also contain an antipor system for monocarboxylates, i.e. glycerate and glycolate, which are both intermediates of the photorespiratory cycle. This transporter will be dealt with in the chapter on photorespiration.

V. Transport of Nucleotides

Plant cells contain a highly active ADP/ATP translocator (AAT) in the inner membranes of mitochondria that exports the ATP generated by oxidative phosphorylation. Heldt (1969) was the first to show that chloroplasts are also capable of transporting ADP and ATP in a counterexchange mode. The proposed physiological function of this translocator in mature leaves is to supply chloroplasts with mitochondrial ATP during the night whereas nongreen plastids depend on the supply with ATP as the driving force for biosynthetic processes, e.g., starch and fatty acids (Hill and Smith, 1991; Kleppinger-Sparace et al., 1992; Neuhaus et al., 1993). A detailed analysis of the kinetic constants of the AAT showed that ATP and ADP are preferentially transported. AMP and other nucleotides showed only a poor interaction with the translocator (Schünemann et al., 1993). It has been claimed that ADP-glucose can also be transported via the AAT into both chloroplasts and amyloplasts (Pozueta-Romero et al., 1991). However, the affinity of AAT toward ADP-glucose is 2 orders of magnitude lower than toward ATP. Because ADP-glucose has to compete with ATP and ADP for binding to the translocator, the amount of ADP-glucose transported by the AAT cannot be of any physiological significance (Schünemann et al., 1993). Nevertheless, a mechanism for the transport of ADP-glucose is required in the endosperm of some cereals. It has been shown recently that in these tissues the AGPase is mainly present in the cytosol but not in the plastids (Denyer et al., 1996; Thorbjørnsen et al., 1996). The cytosolically formed ADP-glucose is presumably transported into the plastids for starch biosynthesis via an ADP-glucose/ADP antiporter which is distinct from the AAT. Most probably, the Bt1 protein, the cDNA of which has already been cloned, serves this function (Sullivan et al., 1991; Sullivan and Kaneko, 1995). The corresponding Bt1 mutation results in an accumulation of ADP-glucose and a reduction of starch biosynthesis in maize, probably reflecting the lack of the ADP-glucose/ADP translocator.

The molecular structure of the plastidic AAT remained elusive until Kampfenkel et al. (1995) happened to isolate a partial cDNA clone from A. thaliana that exhibited similarities to the ADP/ATP translocase from the bacterium Rickettsia prowazekii. Isolation and characterization of the full-length clone revealed no homology to the known mitochondrial adenylate translocators. The corresponding hydrophobic membrane protein (AATP1) possesses an N-terminal region with typical features of plastidic transit peptides. Interestingly, the AATP1 protein contains 12 potential transmembrane helices and thus belongs to the group of transporters with a 12 helix motif as is the case for DiT1 (see above). The transit peptide of AATP1 was able to target the adjacent protein to isolated chloroplasts (Neuhaus et al., 1997). In purified chloroplast envelope membranes, the AATP1 protein (62 kDa) could be detected immunologically. Expression of the AATP1-cDNA in heterologous systems and subsequent transport measurements demonstrates the identity of AATP1 as an ATP translocator. AATP1-specific transcripts could be detected both in photosynthetic and nongreen tissues. Taken together, the results suggest that AATP1 represents the plastidic adenylate translocator that is present in all plant tissues.

VI. Other Translocators

It is well established that chloroplasts are the major site of the synthesis of many amino acids including those of the aspartate and the pyruvate family (Bryan, 1990). In addition, the plastid-located shikimate pathway leads to the formation of the aromatic amino acids phenylalanine, tyrosine and tryptophan. The obvious reason for the chloroplast-located biosynthesis of amino acids is that reduced nitrogen and reducing equivalents, required for some of the biochemical reactions, are produced within the chloroplasts via nitrite reductase and photosynthetic electron transport, respectively, and carbon skeletons (pyruvate (phosphoenolpyruvate), OAA, 2-oxoglutarate) can be imported from the cytosol via specific translocators. The synthesized amino acids are either used for the plastid-located protein biosynthesis or
are, in the main part, exported into the cytosol for intercellular nitrogen transport. Unfortunately, almost nothing is known about plastidic translocators that are involved in these transport processes.

VII. Channels in Chloroplast Envelope Membranes

So far, translocator proteins of the inner chloroplast envelope as the major osmotic barrier between the chloroplast and the cytosol have been dealt with. Both the inner and the outer envelope membrane also contain channels and pores to mediate the flux of ions across these membranes. The outer envelope membrane appears to be permeable to low molecular weight solutes, mediated by the chloroplast porin (Flügge and Benz, 1984). Porins are voltage-dependent channels (VDACs) that are also present in the outer membrane of bacteria and mitochondria (Benz, 1994). In planar bilayers containing reconstituted chloroplast outer envelope membrane proteins, a large conductance channel (\( \Lambda \approx 720 \text{ pS}, \) in 100 mM KCl) was found (Flügge and Benz, 1984). Heiber et al. (1995) reported the presence of a slightly cation-selective high conductance channel (\( \Lambda \approx 1.14 \text{ nS} \) in 100 mM KCl) in the outer envelope membrane. Weak anion and cation-selective large conductance pathways (\( \Lambda \approx 512 \text{ pS} \) and \( \Lambda \approx 1016 \text{ pS} \) in 100 mM KCl) have also been detected in the chloroplast envelope membrane by direct patch-clamping of chloroplasts from the green alga *Nitelopsis obtusa* (Pottosin, 1992, 1993). It has been suggested that the weakly anion-selective channel with a conductivity of \( \Lambda \approx 512 \text{ pS} \) may be related to the mitochondrial VDAC (Pottosin, 1993).

Although the identities of these electrophysiologically characterized channels are not yet known, primary sequences of one class of porins from non-green plastids were recently obtained (Fischer et al., 1994b). These porins showed only a 20-25% identity to the known mitochondrial porins from eukaryotes. However, the plant porins contain 16 antiparallel transmembrane \( \beta \)-strands as is the case for porins from other organisms. The plant porin could be functionally expressed in bacterial cells and the recombinant protein revealed, after reconstitution into planar lipid bilayers, single channel conductances and voltage dependencies almost identical to the authentic protein, 3 and 1.5 nS in 1 M KC1 (Benz et al., 1997). These values are similar to those obtained for plant mitochondrial porins indicating that both non-green plastids and mitochondria possess one type of closely related porin proteins. This conclusion is corroborated by direct alignments of porin sequences from plant mitochondria (Heins et al., 1994).

More recently, the primary sequences of two chloroplast outer envelope membrane proteins were obtained (OEP16, OEP24) that both possess channel-like activities but do not show similarities to bacterial or mitochondrial porins (Pohlmeyer et al., 1997, 1998). Both proteins act as selectivity filters of the outer envelope membrane allowing the passage of amino acids (OEP16) and phosphate, dicarboxylates or adenylates (OEP24), respectively. It is suggested that the outer envelope membrane may contain various channel-like proteins with different selectivities and that the combined action of these channel proteins defines the permeability properties of the chloroplast outer envelope membrane.

Ion channels are also present in the inner envelope membrane. It has already been mentioned that the TPT can function as an anion-selective channel. Ion channel activity of the TPT, however, can only be observed if the concentrations of the monovalent ions are much higher than those of the physiological substrates (Schwarz et al., 1994). Recently, a weakly anion-selective high conductance channel with a single channel conductance of \( \Lambda \approx 525 \text{ pS} \) has been discovered in proteoliposomes containing purified inner envelope membranes from spinach chloroplasts (Fuks and Homble, 1995). A similar weak anion-selective high conductance channel (\( \Lambda \approx 540 \text{ pS} \) was observed by Heiber et al. (1995) in proteoliposomes containing inner and outer envelope membranes from spinach chloroplasts. To prevent uncontrolled dissipation of ion gradients across the inner envelope membrane, these porin-like channels have to be highly regulated.

The inner envelope membrane also contains a chloride channel (Heiber et al., 1995) that might be responsible for the rather high chloride permeability of this membrane (Demmig and Gimmler, 1983). Similar anion-selective channels have been observed in the chloroplast membranes from green algae (Pottosin, 1992; Thaler et al., 1992).

The presence of a potassium channel in the inner chloroplast envelope has been demonstrated by Berkowitz and coworkers (Mi et al., 1994) and Heiber et al. (1995). This K⁺-channel revealed an inward rectifying I/V-relationship, was blocked by millimolar
concentrations of ATP and activated by Mg$^{2+}$ (Heiber et al., 1995). The proposed physiological function of the K$^+$-channel in photosynthetic ion fluxes is outlined in Fig. 5. Light-driven H$^+$-transport into the thylakoids can be counterbalanced by a release of Mg$^{2+}$-ions from the thylakoid lumen. Elevated levels of Mg$^{2+}$- and alkalization of the chloroplast stroma are both required for full activity of the photosynthetic carbon cycle. Stromal Mg$^{2+}$ activates the envelope K$^+$-channel leading to an increased K$^+$-influx that is electrically balanced by H$^+$-efflux via the envelope H$^+$-ATPase. On the other hand, the K$^+$-channel is blocked by increasing concentrations of photosynthetically produced ATP. This effect results in a decreased H$^+$-efflux from the chloroplasts and an acidification of the chloroplast stroma. It is proposed that the opposing modulation of the K$^+$-channel by Mg$^{2+}$-ions and ATP play a major role in the pH-stasis across the chloroplast envelope and the regulation of photosynthesis.

**Fig. 5.** Proposed function of the K$^+$-channel of the chloroplast inner envelope membrane in energy-dependent fluxes of H$^+$ and K$^+$-ions. Modulation of the K$^+$-channel activity by photosynthetically generated ATP and by Mg$^{2+}$-ions takes part in the pH-stasis across the chloroplast envelope during photosynthesis. For details, see text. P$_i$, inorganic phosphate.

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**VIII. Concluding Remarks**

Much progress has been achieved during the last years in studying translocators at biochemical and, more recently, molecular levels. When the corresponding gene is available, its cell- and tissue-specific expression can be studied (e.g., by RNA gel blot analysis, by in situ hybridization, or by expression studies of the corresponding promoter fused to a reporter gene). Furthermore, transgenic plants with a controlled expression of the transporter protein can be used to elucidate the precise role of these translocators in plant metabolism. On the other hand, our knowledge of the identity of other plastidic translocators is still rudimentary. There are multiple reasons for this. First, the biochemical identification and isolation of non-abundant translocators is difficult. Second, the identification of unknown plastidic transporters appears not to be possible using the heterologous complementation system of yeast mutants. As mentioned above, this system has been used extensively to isolate transporters of the plasma membrane. Third, the primary sequences of plastidic transporters known to date revealed only limited homologies to transporters known in other systems. Thus, genes coding for plastidic translocators are rarely obtained by screenings of DNA libraries with heterologous probes.

In the foreseeable future, the *Arabidopsis* and rice genomic sequencing programs will provide the sequences of complete higher plant genomes. In addition, 30,000 *Arabidopsis* ESTs (Expressed Sequence Tags) tagging about half of the expected *Arabidopsis* genes are already available. It appears evident that future work will then concentrate on the identification of the functions of genes coding for putative envelope translocators.
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Chapter 7

Photosynthesis, Carbohydrate Metabolism and Respiration in Leaves of Higher Plants

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Summary

The relationships between photosynthesis, carbohydrate metabolism and respiration in leaves of C_3 plants are reviewed. We first provide an overview of how mitochondrial respiration relies on, and responds to, the supply of photosynthetic products in the light. The pathways by which the various substrates (glycine, oxaloacetate, malate and/or pyruvate) enter the mitochondria and are oxidized, are discussed. We also provide an overview of the pathways of mitochondrial electron transport, with particular attention being paid to the non-phosphorylating alternative oxidase (AOX). We then discuss what is known about leaf respiration rates in light versus darkness (both O_2 consumption and CO_2 release). The extent to which mitochondrial O_2 consumption continues in the light is highly variable, being inhibited, not affected or even stimulated in various reports. On the other hand, non-photorespiratory mitochondrial CO_2 release (R) is invariably inhibited by light (5–80% inhibition). R is sensitive to the lowest irradiance values, and is inhibited rapidly. Three methods via which R in the light is measured are outlined and mechanisms via which light might inhibit R are discussed. The effect that light to dark transitions have on respiration are also discussed: we distinguish the initial, photosynthetic post-illumination burst (PIB) from the post-illumination rise in respiration (LEDR, light-enhanced-dark-respiration) which occurs following the PIB. The chapter also considers the demand for mitochondrially-derived ATP for photosynthesis and carbohydrate metabolism and the potential role of respiration during over-reduction of the chloroplast is highlighted. Mitochondrial respiration appears to be critical for the provision of ATP necessary for energy demanding processes in the light. Moreover, there is growing evidence that respiration helps a plant cope with excess photosynthetic redox equivalents, which otherwise can result in photo-oxidative stress.

Finally, the interactions between nitrogen metabolism, respiration and photosynthesis are also reviewed. Respiration provides the redox equivalents and carbon skeletons necessary for nitrogen assimilation in the light. The inhibitory effect that intermediary nitrogen oxides (NO_x, produced during light to dark transitions) have on mitochondrial electron transport via the cytochrome pathway is discussed.

I. Introduction

Photosynthesis uses light energy and water to convert atmospheric CO_2 into carbon-rich compounds such as carbohydrates. Respiration oxidizes these compounds, releasing CO_2, generating useable energy and forming carbon intermediates needed for biosynthesis. On a whole plant basis, up to 70% of the CO_2 fixed during photosynthesis can be released back into the atmosphere by mitochondrial respiration (Van Der Werf et al., 1994), depending on growing conditions. A thorough understanding of both photosynthesis and respiration, and the interplay between them, is necessary, therefore, if we are to fully understand biomass production in plants.

This chapter describes the relationships between photosynthesis, carbohydrate metabolism and respiration in leaves of C_3 plants. Although leaf mitochondria fulfill important specific functions in C_4 photosynthesis this is outside the scope of this chapter. For this aspect the reader is referred to the excellent review on this topic by Hatch and Carnal.
(1992). We start with an overview of how mitochondrial respiration relies on, and responds to, the supply of photosynthetic products. We then consider what is known about leaf respiration rates in light versus darkness and the mechanisms by which light can affect mitochondrial CO$_2$ release. The demand for mitochondrially-derived ATP for photosynthesis and carbohydrate metabolism is considered and the potential role of respiration during over-reduction of the chloroplast (photo-oxidative stress) is highlighted. The interactions between nitrogen metabolism, respiration and photosynthesis are also reviewed.

II. Supply and Utilization of Mitochondrial Substrates in Leaves

In the light, photorespiration provides glycine as a mitochondrial substrate while carbon fixation in the chloroplast yields triose-phosphate in the cytosol, which, after conversion to phosphoenolpyruvate (PEP), can subsequently be converted either to pyruvate via pyruvate kinase (PK) or oxaloacetate (OAA) via PEP carboxylase (Fig. 1). The OAA may be reduced to malate in the cytosol if sufficient NADH is available. These substrates can all be oxidized in mitochondria. Pärnik and Keerberg (1995) defined such substrates as primary products of photosynthesis/photorespiration. The major primary photosynthetic product used as a substrate by mitochondria in the light appears to be glycine, while products from PEP represent ca. 3–15% of primary substrates that are oxidized (Ellsworth and Reich, 1996; Hurry et al., 1996). The time taken for these substrates to be exported from the chloroplast to the respiratory apparatus is likely to be in the order of seconds to minutes. For example, the period between Rubisco oxygenation in the chloroplast and the initiation of photorespiratory mitochondrial CO$_2$ release in tobacco is approximately 15–20 s (Atkin et al., 1998a).

Starch and sucrose generated by photosynthetic carbon fixation minutes, hours or days previously can also serve as respiratory substrates in leaves. Such photosynthetic end-products (Ellsworth and Reich, 1996) can represent 40–50% of total substrate oxidation in the light (Ellsworth and Reich, 1996; Hurry et al., 1996). In the presence of sufficient Pi, starch degradation in the light can occur at rates comparable to the rate of starch synthesis (Stitt and Heldt, 1981). Darkness eliminates the production of primary carbon products and respiration then relies completely on degradation of starch and sucrose reserves.
A. Export of Carbon From Chloroplasts

Carbon is exported from the chloroplasts mainly as triose-phosphates on the phosphate/triose-phosphate exchange translocator (Flügge and Heldt, 1991). In the cytosol, triose-phosphates can be further oxidized through glycolysis or combined to form fructose 1,6 bisphosphate (F1,6BP). Subsequent dephosphorylation produces hexose phosphates which serve as substrates for gluconeogenesis or for oxidation and decarboxylation via the pentose phosphate pathway. The synthesis of sucrose is regulated by the availability of UTP for UDP-glucose synthesis and also by the phosphorylation state of sucrose phosphate-synthase (SPS) which can be modulated allosterically by changes in G6P and Pi concentrations (Krömer, 1995). The sucrose formed can be transported to other parts of the plant, or stored locally and subsequently degraded by invertase or sucrose synthase for entry into oxidative pathways. These reactions are summarized in Fig. 1. Of the hexose phosphates oxidized, approximately 70–95% are degraded via phosphofructokinase and aldolase in glycolytic reactions; the remainder are oxidized to triose-phosphates via the OPP pathway (ap Rees, 1980).

B. Products of Glycolysis

Triose-phosphates are oxidized through glycolysis, yielding ATP and NADH, to form PEP. PEP carboxylase and PK compete for cytosolic PEP and both reactions are essentially irreversible. The activity of both PEP carboxylase and PK can be finely controlled, allowing the plant cell to regulate the availability of OAA, malate and pyruvate for oxidation by mitochondria. PK is inhibited by high concentrations of ATP (Hatzfeld and Stitt, 1991; Podstá and Plaxton, 1994) but is activated by dihydroacetone-3-phosphate (DHAP) in algal cells (Lin et al. 1989). In contrast, PEP carboxylase activity is not regulated by the cell’s energy status but is inhibited by malate, aspartate and glutamate (Lepiniec et al., 1994; Krömer et al., 1996). The degree of inhibition can be regulated by phosphorylation of PEP carboxylase by a protein kinase (Van Quy et al., 1991), with the phosphorylated form being less sensitive to inhibition by metabolites (Krömer et al., 1996). PEP carboxylase activity is also increased by G6P, DHAP, 3-phosphoglycerate (3-PGA) and inorganic phosphate (P_i) (Krömer et al., 1996). At physiological concentrations of malate and G6P, the activation by G6P overrides malate inhibition (Krömer et al., 1996). The regulatory properties of PEP carboxylase may be summarized as a feed-forward activation by primary products of photosynthetic carbon fixation and a feed-back inhibition from malate and amino acids.

The ability of the PEP carboxylase route to supply OAA and/or malate to the mitochondria is shown by transgenic PK-deficient tobacco plants which exhibit a phenotype not significantly different from wildtype plants (Gottlob-McHugh et al., 1992). The question of whether PEP carboxylase activity results in OAA or malate being imported into the mitochondria, depends on whether OAA is converted to malate by cytosolic MDH (which may happen when the cytosolic NADH/NAD ratio is high).

C. Export of Redox Equivalents from Chloroplasts

Photosynthetic redox equivalents can be exported from the chloroplast to the cytosol in the form of triose-phosphates via the DHAP:3-PGA shuttle in the inner envelope of the chloroplast (Heber, 1975; Heineke et al., 1991). If the export of DHAP is coupled to the synthesis of 3-PGA in the cytosol, then one molecule of both ATP and NADH, or NADPH alone, will be liberated into the cytosol for each molecule of DHAP exported, depending on the cytosolic enzymes that are involved (Krömer, 1995). 3-PGA can be imported subsequently into the chloroplast thus completing the cycle. This shuttle mechanism appears to be controlled by the relative rates of chloroplastic 3-PGA reduction and cytosolic DHAP oxidation (Heineke et al., 1991).

Reductants can also be exported from the chloroplasts via malate/OAA exchange (Fig. 2). Chloroplasts contain a NADP+-malate dehydrogenase (NADP+-MDH) which is light inducible (Scheibe, 1987). Under conditions where the NADPH/NADP+ ratio in the chloroplast is high (Scheibe, 1987; Heineke et al., 1991), OAA will be converted to malate. Malate can subsequently be exported via the malate-OAA translocator present in the inner envelope membrane of the chloroplast (Hatch et al., 1984). It has been suggested that this shuttle allows excess photosynthetic redox equivalents to be exported from the chloroplast in the form of malate (Heineke et al., 1991). The operation of the shuttle appears to be driven by a redox gradient between the chloroplast and the cytosol, which will be substantial...
in the light. For example, the ratio of NADPH:NADP⁺ in the stroma of spinach chloroplasts is approximately 0.5, whereas the NADH:NAD⁺ ratio in the cytosol is far lower (0.001; Heineke et al., 1991). Since the chloroplast NADP⁺-MDH is only active at high stromal NADPH/NADP⁺, and is inactivated in the dark, the shuttle only operates in the light when there is excess reductant available. In this way NADP⁺-MDH in the chloroplast acts as a ‘redox valve’ (Kröamer and Scheibe 1996). In addition to being controlled by redox gradients between the chloroplast and the cytosol, malate export also is regulated by the translocating step across the envelope membrane (Heineke et al., 1991). The malate thus released into the cytosol could either:

1) be oxidized in peroxisomes to support hydroxypyruvate reductase during operation of the photorespiratory cycle;

2) be oxidized in mitochondria, allowing ATP to be synthesized (which may be important for rapid sucrose synthesis—see below); or

3) be oxidized in the cytosol to support nitrate reduction.

In all cases, the OAA formed in the cytosol must return to the chloroplast to complete the shuttle. Figure 2 illustrates these reactions.

D. Oxidation of Organic Acids by Mitochondria

1. Pathways of Oxidation

Pyruvate, malate and OAA can be imported into the mitochondrial matrix via carriers present on the inner mitochondrial membrane (Day and Wiskich, 1984; Fig. 3). Malate uptake could occur via the dicarboxylate carrier which catalyses malate/Pi exchange. On the other hand, plant mitochondria also contain a specific transporter which catalyses OAA uptake in exchange for either malate or citrate (Heldt and Flügge 1987; Hanning et al., 1999), and this transporter may be involved in transfer of reductant and carbon skeletons out of the mitochondrion (Woo and Osmond, 1976; Day and Wiskich, 1981; Zoglowek et al., 1988).

Malate is present in high concentrations in leaves (although much of it is in the vacuole) (Gerhardt and Heldt, 1984; Heineke et al., 1991) and can serve as an important respiratory substrate (Lance and Rustin, 1984). Malate levels increase during photosynthesis (Gerhardt et al., 1987; Heineke et al., 1991; Xue et al., 1996), as a consequence of both the export of redox equivalents from the chloroplast via the malate/
OAA shuttle (Heineke et al., 1991) and PEP carboxylase/MDH activity (Hill and Bryce, 1992). Martinoia and Rentsch (1994) concluded that because cellular concentrations of malate decrease in the dark, vacuolar malate is likely to be used by the TCA cycle as a substrate. However, the capacity for malate import into isolated mitochondria appears to be considerably lower than the capacity for OAA import (Zoglowek et al., 1988; Krömer, 1995) and OAA is potentially an alternative substrate for mitochondria. The greater activity of the OAA transporter may be off-set in vivo by the very low concentration of this metabolite in the cytosol.

If malate is imported, it can be converted both to OAA by MDH and to pyruvate via NAD-ME. The same enzymes can make pyruvate from OAA if the matrix NADH concentration is high. The advantage of producing both substrates within the mitochondrion is that carbon intermediates can be exported from the TCA cycle; for example, citrate or 2-oxoglutarate can be exported to support GOGAT activity in the chloroplast (Fig. 3). Pyruvate can also be imported into the mitochondrion, to feed into a complete or partial TCA cycle (Fig. 3).

2. Mitochondrial Electron Transport

Partial or complete operation of the TCA cycle yields NADH which is oxidized by the respiratory chain to produce ATP (Fig. 4). The amount of ATP formed will depend on the activity of the non-phosphorylating by-passes of the respiratory chain.

Two NADH-oxidizing enzymes are available from the matrix of mitochondria. A rotenone-insensitive NADH dehydrogenase exists on the matrix side of the inner mitochondrial membrane in plants, which does not pump protons and bypasses the energy-conserving complex I (Fig. 4). The activity of this bypass appears to be largely governed by the concentration of NADH in the matrix, since it has a significantly lower affinity for NADH than does complex I (Møller and Lin, 1986). When substrate supply to the mitochondrion is high, the bypass will contribute to NADH oxidation and decrease ATP production rates. These conditions (high matrix NAD(P)H, high internal pyruvate concentration and reduced ubiquinone) will also lead to activation of the non-phosphorylating alternative oxidase (AOX) of the plant respiratory chain, further decreasing ATP production. These two bypasses of the proton translocating respiratory complexes could allow leaf cells to oxidize mitochondrial NADH when cytosolic adenylate levels limit the cytochrome path (or it is overwhelmed) and may act as safety valves, preventing over reduction of the mitochondrial NAD and ubiquinone pools and avoiding generation of

AOX activity appears to be regulated by a sophisticated feed-forward activation mechanism. AOX exists as a dimer which under oxidizing conditions is covalently linked by disulfide bonds (Umbach and Siedow, 1993; Umbach et al., 1994). The AOX protein must be reduced in order to be activated (Umbach and Siedow, 1993), probably via matrix NADPH in a thioredoxin-mediated reaction (Vanlerbergh et al., 1995; Day and Wiskich, 1995). This reduction process may involve the interconversion of S-S to -SH groups in a particular cysteine residue on the protein, although this has not been conclusively demonstrated. The reduced form of AOX can be stimulated by μM concentrations of pyruvate and some other 2-oxo acids, such as glyoxylate (Millar et al., 1993; Day et al., 1995; Millar et al., 1996). The substrate for AOX is ubiquinol (Qr). Stimulators such as pyruvate appear to alter the interaction between Q and AOX, so that the oxidase is active at lower levels of reduced Qr. The cytochrome and alternative oxidases have been shown to compete for electrons from Q when AOX is activated by pyruvate (Hoefnagel et al., 1995; Ribas Carb et al., 1995).

Not all species express AOX constitutively in their leaves (in such plants AOX synthesis is usually triggered by environmental stress: McIntosh, 1994). In those that do, the oxidase is likely to be active under photosynthetic conditions when the cell redox pools are highly reduced, particularly in combination with plentiful supply of substrates and metabolites such as pyruvate and glyoxylate. The latter may be an important feed-forward activator during photorespiration. Recent studies of respiration in a period following illumination indicate that AOX is activated in the light (Igamberdiev et al., 1997a). There is also some evidence that AOX synthesis and contribution to respiratory oxygen uptake increases in photosynthetic organs during greening. For example, exposure of etiolated Belgium endives to light causes induction of cyanide-insensitive respiration within 24 h (Atkin et al., 1993). In soybeans, measurements using the non-invasive 14O discrimination technique (Guy et al., 1992) have shown that AOX contribution to dark respiration increases during greening of cotyledons (Ribas-Carb et al., 1997; Robinson et al., 1995) and the amount of AOX protein also increases (Finnegan et al., 1997).

**E. Glycine Oxidation During Photorespiration and Interactions with the TCAC**

The photorespiratory glycolate cycle provides a major mitochondrial substrate in photosynthetic tissues. Previous chapters have outlined how under ambient atmospheric conditions P-glycolate is produced via the oxygenase reaction of Rubisco in the chloroplast: this oxygenase reaction accounts for 20–35% of the net photosynthetic activity (Lorimer and Andrews, 1981; Dry et al., 1987). P-glycolate is converted to glycolate and exported to the peroxisome, where the glycolate is converted to glycine; this in turn is exported to mitochondria to be oxidized as a respiratory substrate.

The degree to which photorespiration contributes to mitochondrial electron transport (and thus ATP synthesis) will depend on the degree to which NADH produced by glycine decarboxylation is exported to the cytosol (via the OAA-malate shuttle mechanism) or oxidized within the mitochondria. Maintenance of
the photorespiratory cycle requires that equal amounts of NADH be reoxidized in the peroxisome (during the conversion of OH-pyruvate to glycerate) as is released during mitochondrial glycine decarboxylation (Leegood et al., 1995). In vivo, it is likely that at least some of the NADH produced by glycine oxidation is oxidized in the peroxisome, since the malate/OAA shuttle capacity in isolated mitochondria exceeds the capacity of GDC (Ebbighausen et al., 1987). However, if the peroxisome requirements for NADH can be met partly by glycolysis or the plastid (see above), then oxidation of glycine can contribute to mitochondrial ATP synthesis. In experiments with isolated mitochondria under simulated in vivo conditions, Krömer et al., (1992) estimated that 25%–50% of NADH from glycine oxidation was shuttled out of the mitochondria. In line with this, the cytosolic ATP/ADP ratio in barley protoplasts was higher under photorespiratory than in non-photorespiratory conditions (Gardeström and Wigge, 1988).

In vivo, it is probable that photorespiration and the TCA cycle operate side-by-side. However, it is imperative that glycine oxidation in the mitochondria proceeds as rapidly as possible to prevent accumulation of toxic intermediates in the photorespiratory cycle. In isolated leaf mitochondria, glycine oxidation takes precedence over the oxidation of other mitochondrial substrates (Dry et al., 1983; Day et al., 1985; Igamberdiev et al., 1997b). This preferential oxidation of glycine is partly achieved by a dominance of complex I over both complex II (SDH) and the external NADH dehydrogenase of the respiratory chain, ensuring that matrix NADH is rapidly oxidized (Day et al., 1985). GDC also effectively competes against the TCA cycle dehydrogenases for NADH in the matrix (Day et al., 1985). TCA cycle activity may, therefore, be limited under photorespiratory conditions by NADH availability and inhibition of succinate oxidation. Measurements on barley protoplasts have shown that the matrix NADH/NAD pool is more reduced under photorespiratory conditions than in non-photorespiratory conditions (Wigge et al., 1993).

Concurrent oxidation of malate and glycine poses other problems for leaf mitochondria if GDC is linked to a malate/OAA shuttle to the peroxisome; under these conditions malate must be both taken up and exported from the mitochondria. This dilemma could be solved by separate but simultaneous operation of the dicarboxylate and OAA carriers. However, under these conditions mitochondrial MDH must function simultaneously in different directions (malate > OAA in the TCA cycle and OAA > malate in the shuttle). A study of pea leaf mitochondria by Wiskich et al. (1990) points to a possible solution. These authors suggested that the mitochondrial matrix consists of metabolic domains in which groups of MDH enzymes are associated in discrete protein complexes termed ‘metabolons.’ Substrate channeling in these metabolons would allow MDH to operate simultaneously in opposite directions, within the same mitochondrion. Alternatively, OAA could be the main substrate taken up by the mitochondria. An OAA_{in}/malate_{out} exchange could shuttle excess redox equivalents out of the mitochondria and a fraction of OAA could condense with acetylCoA (formed from pyruvate either taken up from the cytoplasm or produced from malate by ME). In such a scheme MDH would only operate in the OAA > malate direction and the uptake of OAA would be linked to either malate or citrate export.

The concept of metabolons offers a possible explanation for the continued operation of the TCA cycle in the face of large amounts of photorespiratory NADH produced in the light in the mitochondrial matrix. High NADH levels can restrict activity of the various TCA cycle dehydrogenases (Dry and Wiskich, 1985) but kinetic separation of the GDC-MDH complex from the other TCA cycle enzymes might reduce this inhibitory potential.

III. Mitochondrial Function in the Light and Dark

Light increases the cellular demand for TCA cycle carbon skeletons (e.g., for ammonia assimilation), NADH (e.g., for the reduction of NO\textsubscript{2} in the cytosol and OH-pyruvate in the peroxisomes) and ATP (e.g., for NH\textsubscript{3} assimilation and sucrose synthesis), in leaf cells. Moreover, decarboxylation of glycine to serine releases substantial amounts of CO\textsubscript{2} and potentially contributes to O\textsubscript{2} consumption. All of this suggests that respiration rates might be greater in the light than in the dark; yet measurements of respiratory CO\textsubscript{2} release often suggest the opposite. In this section we assess the response of mitochondrial CO\textsubscript{2} release and O\textsubscript{2} uptake in leaves in the light.

Measurements of respiratory gas exchange in the dark are relatively straightforward, but the same cannot be said for measurements of respiration in the
light. In the light, photorespiratory and non-photorespiratory reactions result in mitochondrial O₂ consumption, while O₂ is produced via photosynthesis. Moreover, O₂ is also consumed in the chloroplast as a result of Rubisco oxygenation and the Mehler reaction. While definitive measurements of Mehler reaction rates are lacking, available data suggests that it does not contribute significantly to O₂ consumption in the light in leaves of some plants (Peltier and Thibault, 1985) and in algae (Xue et al., 1996). Determinations of TCA cycle CO₂ release in the light are complicated by the presence of other CO₂ releasing and assimilating reactions. For example, in the light CO₂ is fixed by Rubisco and PEP carboxylase, whereas CO₂ is released by glycine decarboxylation, the TCA cycle and the OPP pathway. Measurements of respiratory activity, therefore, need to account for each of the above processes.

A. Mitochondrial O₂ Consumption in the Light

Estimates of mitochondrial O₂ consumption in the light often yield conflicting results and have indicated it to be unaffected, inhibited or even stimulated (Turpin and Weger, 1989). For example, ¹⁶O₂/¹⁸O₂ analysis of oxygen exchange in Dunaliella tertiolecta indicated that mitochondrial O₂ consumption is inhibited by light (Bate et al., 1988; Canvin et al., 1980). In contrast, similar analysis in carnation cells has indicated that mitochondrial O₂ consumption is similar in the light and dark (Peltier and Thibault, 1985; Avelange et al., 1991), and several studies have reported that light increases mitochondrial O₂ consumption in green algae (Xue et al., 1996).

This conflict in mitochondrial response to light probably reflects the variability in provision of carbon from photosynthesis, the degree to which photorespiratory NADH is consumed in the mitochondria, and the degree to which excess photosynthetic redox equivalents are exported from the chloroplast. These factors in turn are likely to vary with the experimental conditions employed (especially light intensity and temperature). In green alga, the rate of mitochondrial O₂ consumption in the light is greater at high irradiance values than at low irradiance (Xue et al., 1996). Xue et al. (1996) concluded that export of excess photosynthetic redox equivalents provides NADH to the mitochondria in an irradiance-dependent manner. Taken together, the available studies suggest that mitochondrial O₂ consumption can indeed be maintained or even stimulated in the light, although substantial genetic and environmental variation can occur.

B. Mitochondrial CO₂ Release in the Light

1. ¹⁴C-Labeling Measurements

Under photorespiratory conditions (e.g., 21% O₂, 350 ppm CO₂), total mitochondrial CO₂ release is greater in the light than darkness due to the additional CO₂ release associated with glycine decarboxylation. However, there is growing evidence that non-photorespiratory mitochondrial CO₂ release (R) is inhibited in the light.

Using a ¹⁴C-labeling pulse-chase technique, Pärnik and Keerberg (1995) demonstrated that R is lower in the light than in darkness in several species. This method rests on the assumption that respiration is not affected by decreasing the concentration of oxygen from 21% to 2%. It is able to distinguish between four components of CO₂ release in leaves, depending on the nature of the carbon compounds produced (primary products such as triose-P, malate and glycine released rapidly from the chloroplast vs. secondary end products such as sucrose and starch: see Section II) and the decarboxylation pathway employed to release the CO₂ (i.e. photorespiratory vs. (non-photo)respiratory).

Pärnik and Keerberg (1995) reported that respiratory decarboxylation of end products in the leaves in the dark was 0.64, 1.27 and 0.6 µmol CO₂ m⁻² s⁻¹ for wheat, tobacco and barley, respectively. In the light, respiratory decarboxylation of these products was reduced by 60–70% (to 0.27, 0.45 and 0.23 µmol CO₂ m⁻² s⁻¹ for wheat, tobacco and barley, respectively (Pärnik and Keerberg, 1995). This decrease was partly offset by the respiratory decarboxylation of photosynthetic primary products, which amounted to 0.28, 0.23 and 0.04 µmol CO₂ m⁻² s⁻¹ in the light, respectively for the three species above. Consequently, the total rate of respiratory decarboxylation in the light was 0.55, 0.68 and 0.27 µmol CO₂ m⁻² s⁻¹ for wheat, tobacco and barley. Light, therefore inhibited total oxidative decarboxylation by 14, 46 and 55% in wheat, tobacco and barley, respectively (Pärnik and Keerberg, 1995).

The inhibition of total respiratory decarboxylation in these species apparently resulted from a combination of lower rates of non-photorespiratory decarboxylation of end products in the light and relatively low rates of primary product use (or
provision). Separate experiments using $^{14}$C labeled carbon have confirmed that $R$ is inhibited by light in wheat (McCashin et al., 1988). However, light was found to stimulate total respiratory decarboxylation in winter rye by 31% (Hurry et al., 1996), showing that light does not necessarily inhibit $R$ in all species.

2. Laisk Method Measurements

The hypothesis that light inhibits $R$ is supported by studies using the Laisk (1977) method for measuring $R$. For example, light inhibits $R$ in spinach, barley, tobacco, *Eucalyptus* sp., *Poa* spp., as well as in evergreen and deciduous shrubs (Brooks and Farquhar, 1985; Kirschbaum and Farquhar, 1987; Atkin et al., 1998a,b; Villar et al., 1994; 1995). The Laisk method analyzes the rate of net CO$_2$ exchange ($A$) at low internal CO$_2$ concentrations ($c_i$) and varying irradiances. $A$ is related to $R$ in the light ($R_o$) according to:

$$ A = v_c - 0.5v_o - R_d $$

where $v_c$ and $v_o$ are the rates of carboxylation and oxygenation of Rubisco, respectively. Photorespiration results in one molecule of CO$_2$ released for each two molecules of O$_2$ that are consumed by Rubisco. Figure 5 shows how decreases in $c_i$ lead to decreased CO$_2$ fixation ($v_c$) and increased CO$_2$ release (0.5 $v_o$). Decreasing $c_i$ eventually results in the CO$_2$ fixed by Rubisco being matched by the CO$_2$ that is released by glycine decarboxylation: i.e., $v_c$ and 0.5 $v_o$ are equivalent. This point is termed $\Gamma_s$. $R_d$ is the rate of CO$_2$ release at $\Gamma_s$. Non-photorespiratory mitochondrial CO$_2$ release can be measured, therefore, by exposing illuminated leaves to $\Gamma_s$ and measuring the net assimilation rate ($A_{net}$).

To determine both $\Gamma_s$ and $R_d$, regressions of $A_{net}$ versus $c_i$ at different irradiances must be constructed, as shown in Fig. 6 (Atkin et al., 1997). These regressions intersect at a $c_i$ equal to $\Gamma_s$, and $R_d$ is the rate of CO$_2$ exchange at that $c_i$. The irradiance values used to construct such regressions should be at least 80 $\mu$mol photons m$^{-2}$ s$^{-1}$, as $R_d$ decreases sharply at lower irradiance values (Brooks and Farquhar, 1985; Atkin et al., 1998a).

The Laisk method assumes that $R_d$ remains constant across a wide range of $c_i$ values. It also assumes that $R_d$ is not substrate limited during prolonged exposure to low CO$_2$ concentrations. Figure 5 demonstrates that CO$_2$ uptake by Rubisco is severely limited at $\Gamma_s$.

![Fig. 5. Modeled CO$_2$ exchange (µmol CO$_2$ m$^{-2}$ s$^{-1}$) versus CO$_2$ internal concentration ($c_i$, ppm) for tobacco leaves. The thin solid line represents the rate of CO$_2$ uptake by Rubisco (i.e., $v_c$), the dotted thin line represents the rate of photorespiratory CO$_2$ release (i.e., 0.5$v_o$), and the thin hashed line represents the rate of non-photorespiratory CO$_2$ release ($R_d$). The thick solid line represents the net assimilation rate (i.e., $v_c - 0.5v_o - R_d$). Data for this figure was kindly provided by Dr. J. Evans, RSBS, ANU.](image)

Hence, supply of primary photosynthetic substrates to the mitochondria could potentially become limiting with time. However, experiments using a fast-response gas exchange system to rapidly expose tobacco leaves to $\Gamma_s$, following a period of photosynthesis at 350 ppm CO$_2$, have recently demonstrated that $R_d$ is not substrate limited at $\Gamma_s$ (Atkin et al., 1998a).

3. Kok Method Measurements

Another method for determining the rate of $R$ in the light is the Kok (1948) method. This approach requires that net CO$_2$ exchange be measured at a set CO$_2$ partial pressure and at several irradiance levels, as shown in Fig. 7. Decreases in irradiance (starting at 100 $\mu$mol photons m$^{-2}$ s$^{-1}$) initially result in linear decreases in net assimilation (Fig 7). However, there is a break in this linear response as net assimilation approaches zero, with the slope increasing markedly at lower irradiance levels. The measured rate of gas exchange at zero irradiance is $R_n$ (shown as the
Estimates of $R$ in the light ($R_d$) are obtained by extrapolating the linear section down to zero irradiance (i.e., the intercept with the axis of net assimilation, shown as an open square). The break from linearity is most likely the result of $R$ no longer being inhibited by light at low irradiance levels: as irradiance decreases, the degree of light inhibition of $R$ also decreases. The irradiance at which the plot breaks from linearity provides an estimate of the irradiance at which $R$ is fully inhibited by light. Several studies have used the Kok method to demonstrate that is lower than (Freyer and Wiesberg, 1975; Kirschbaum and Farquhar, 1987; Sharp et al., 1984; Villar et al., 1994).

One problem with the Kok method is that the decrease in irradiance during the measurements can result in a gradual increase in $c_i$ (which suppresses photorespiration and increases carboxylation) and a concomitant relative increase in the rate of net assimilation in the linear region (Villar et al., 1994). As a result, the slope of the linear region plotted through observed data is less that it would be if $c_i$ had stayed constant at the values indicated. The three dashed lines emanate from the true $R_d$ value (as shown) which is more negative than the apparent $R_d$ value estimated from the solid line (Kirschbaum and Farquhar, 1987). The Kok method can, therefore, result in underestimates of the true $R_d$ value. Theoretically, the Kok effect (break from linearity) could be seen even when $R_d$ and $R_n$ are identical (due to the slope at higher irradiances being affected by the changing $c_i$). The true $R_d$ value can be obtained by correcting the apparent $R_d$ value to a constant $c_i$ using the program reported by Kirschbaum and Farquhar (1987).

Villar et al. (1994) compared the Kok and Laisk methods. They found the Kok method consistently resulted in lower estimates of $R_d$ even following correction for changing $c_i$. Moreover, estimates of $R_d$ obtained using the Laisk method were more consistent than estimates using the Kok method and it is sometimes very difficult to detect the change in slope that occur during the Kok measurements. Kok measurements of $R_d$ can take over five hours to complete, whereas measurements of $R_d$ obtained with the Laisk method are complete within two
hours (Villar et al., 1994). It is therefore preferable to use the Laisk method to estimate $R_{at}$, but both methods have indicated that mitochondrial CO$_2$ output is decreased in the light in leaves.

C. Effect of Light to Dark Transitions on Respiration

Illuminated leaves often exhibit large transient increases in O$_2$ consumption and CO$_2$ release when first exposed to darkness (Fig. 9). This rapid increase is termed the photorespiratory post-illumination burst (PIB). It is followed by a second slower rise in $R$, which is termed light-enhanced-dark-respiration (LEDR). Some post-illumination studies have incorrectly assigned PIB to increases in $R$ that are clearly LEDR in nature. The two peaks in respiration occur at separate times, differ in magnitude and are caused by quite separate physiological events.

The PIB peak represents CO$_2$ release by photorespiratory glycine decarboxylation. While RuBP carboxylation decreases immediately after darkening, glycine decarboxylation does not begin to decrease for several seconds (presumably due to the time taken for photorespiratory products to reach the mitochondria). Usually, the PIB peak is seen about 20 s after the light is switched off (Bulley and Tregunna, 1971; Doehlert et al., 1979; Atkin et al., 1998a,b). The idea that PIB is photorespiratory in origin is supported by the fact that this peak does not occur in leaves exposed to low O$_2$ (e.g., 2%; Fig 9) or high CO$_2$ concentrations that inhibit the oxygenation reaction of Rubisco (Bulley and Tregunna, 1971; Doehlert et al., 1979; Atkin et al., 1998a). The size of the PIB peak is increased under conditions of high glycolate production (low CO$_2$, high light: Doehlert et al., 1979; Atkin et al., 1998a).

LEDR, on the other hand, reflects an increased supply of non-photorespiratory substrates (e.g. pyruvate and/or malate) which accumulate during photosynthesis (Raghavendra et al., 1994). Although LEDR is often detected as increased O$_2$ consumption (Azcon-Bieto et al., 1983; Reddy et al., 1991; Gardeström et al., 1992; Hill and Bryce, 1992; Xue et al., 1996; Igamberdiev et al., 1997a), transient increases in CO$_2$ evolution have also been reported (Xue et al., 1996; Atkin et al., 1998a,b). The magnitude of the LEDR peak is increased when photorespiration is decreased under low O$_2$ (Atkin et al., 1998a; Igamberdiev et al., 1997a). The magnitude of LEDR also increases with increasing irradiance during the preceding light period (Atkin et al. 1998a,b). LEDR therefore appears to reflect the level of photosynthetetic metabolites available to the mitochondria following a period of illumination (Hill and Bryce, 1992; Xue et al., 1996; Igamberdiev et al., 1997a; Atkin et al., 1998a,b; Padmasree and Raghavendra, 1998).
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D. Mechanisms Responsible for LEDR

The mechanism leading to sustained, enhanced respiration after a period of illumination has not been determined, but the results of Hill and Bryce (1992) suggest that LEDR may result from an increase in malate oxidation after illumination. Malate pools in leaf cells are high at the commencement of the dark period (Hill and Bryce, 1992; Xue et al., 1996), and the activity of NAD-ME and PDC increase in the dark. Consequently, malate concentrations decrease during lengthy dark periods and respiration rates fall concurrently (Hill and Bryce, 1992; Xue et al., 1996). The corollary of this is that inactivation of ME and PDC underlies the decrease in $R$ in the light (see below).

The mechanism responsible for the light inhibition of NAD-ME is unresolved. The enzyme remains inactive during isolation of mitochondria from illuminated leaves and subsequent gel filtration of mitochondrial extracts. There is no evidence that NAD-ME can be phosphorylated, but other covalent modifications, or altered binding of cofactors, could be involved (Cook et al., 1995). Leaf mitochondrial PDC, on the other hand, can be reversibly inactivated in the light (Randall et al., 1990), due to phosphorylation of the enzyme (Randall and Budde, 1987; Schuller and Randall, 1989). The inhibition of PDC activity mainly occurs under photorespiratory conditions (Budde and Randall, 1990; Gemel and Randall, 1992). For example, exposure of pea leaves to 1500 ppm CO$_2$ (to inhibit photorespiration) results in a 60% to 80% decrease in the light-dependent inactivation of PDC (Gemel and Randall, 1992). The photorespiration-dependent inhibition of PDC may be due to NH$_3$ (produced during glycine decarboxylation) stimulation of the protein kinase which phosphorylates PDC (Budde and Randall, 1987; Krömer, 1995). Increased ATP synthesis due to increased electron transport during glycine oxidation could also contribute to PDC inactivation (Moore et al., 1993). In this context, Lernmark and Gardeström (unpublished) found that the degree of photorespiration-dependent inactivation of PDC is less under conditions which decrease mitochondrial ATP synthesis. The light dependent inactivation of PDC has been reported in pea, zucchini, soybean, barley, tobacco and maize (Gemel and Randall, 1992). However, other studies of barley protoplasts showed no inactivation of PDC under photorespiratory conditions (Krömer et al., 1994). The difference between species might simply reflect varying degrees of contamination with plastid PDC isoenzymes which are not inactivated by phosphorylation (Lernmark and Gardeström, 1994).

E. Relationship Between Light Inhibition of Mitochondrial CO$_2$ Release ($R$) and LEDR

The mechanism by which $R$ is inhibited in the light remains to be elucidated. However, it is interesting that both LEDR and light inhibition of $R$ are equally sensitive to increasing irradiances in the light period (Atkin et al., 1998a). $R$ is inhibited at irradiance values as low as 3 μmol photons m$^{-2}$ s$^{-1}$ (Brooks and Farquhar, 1985; Atkin et al., 1998a,b) and is also the lowest irradiance which results in significant LEDR (Atkin et al., 1998a). Moreover, both parameters are insensitive to light quality (Xue et al., 1996; Atkin et al., 1998a) and are tightly correlated (Atkin et al., 1998a,b). It is possible, therefore, that both phenomena have a common cause, and the Hill and Bryce (1992) model for LEDR may also provide a mechanism for inhibition of $R$ in the light. According to this hypothesis, inhibition of $R$ in the light results from limited flux through ME and PDC. Inactivation of PDC alone by light would limit the conversion of pyruvate to acetyl CoA (and thus TCA cycle CO$_2$ release), regardless of whether the pyruvate resulted from the action of ME or pyruvate kinase (PK). Coordinated inactivation of both ME and PDC would further strengthen the inhibitory effect light has on pyruvate flux into the TCA cycle, especially in tissues where malate is an important carbon source for the mitochondria (see below). Inactivation of ME/PDC might also explain why light inhibits $R$ so rapidly (within 50 seconds; Atkin et al., 1998a,b): the timing of inactivation of ME (Hill and Bryce, 1992) closely mirrors the 50 s taken for light to fully inhibit $R$ (Atkin et al., 1998a,b). Measurements of the irradiance dependence of ME and PDC activation state are needed to test this hypothesis.

F. Role of Nitrogen Assimilation in Light Inhibition of $R$

Enhanced export of TCA cycle carbon intermediates to the cytosol during light-dependent nitrogen assimilation may also contribute to inhibition of $R$ in the light. TCA cycle CO$_2$ release would be substantially reduced under conditions where 2-oxoglutarate and/or citrate are removed to support...
amino-acid synthesis. In the absence of increased flux of carbon into the TCA cycle, removal of citrate or 2-oxoglutarate would eliminate one site of \( \text{CO}_2 \) release. Support for this suggestion comes from the fact that the \( \text{CO}_2 \) compensation point of barley leaves increases when plants are transferred from \( \text{NO}_3 \) to \( \text{NH}_4 \) nutrient (Faire et al., 1972). \( \text{NH}_4 \) is not transported from roots to shoots but rather is assimilated in the roots. As a result, transfer to \( \text{NH}_4 \) growth media would largely eliminate leaf N-assimilation and thereby decrease the demand for TCA cycle intermediates in the leaves. This in turn could result in an increase in TCA cycle \( \text{CO}_2 \) release and the \( \text{CO}_2 \) compensation point. Further work is required to test this hypothesis.

**G. Mitochondrial Electron Transport and ATP Production in the Light**

The fact that photosynthesis itself results in ATP synthesis has led several authors in the past to suggest that mitochondrial electron transport might be more limited by high ATP/ADP ratios in the light than in the dark (Graham, 1980; Villar et al., 1995). However, this does not appear to be the case. At saturating \( \text{CO}_2 \) concentrations, cytosolic ATP/ADP ratios are lower in the light than in darkness (Hampp et al., 1982; Stitt et al., 1982; Krömer, 1995). While cytosolic ATP/ADP ratios are higher in the light than in darkness when \( \text{CO}_2 \) limits photosynthesis (Gardeström, 1987; Krömer, 1995), they are not sufficiently high to result in adenylate restriction of respiration (Krömer, 1995). Krömer (1995) concluded that, despite some contribution by photophosphorylation, most of the cytosolic ATP pool is maintained by mitochondrial oxidative phosphorylation. Indeed, respiratory chain activity in the light appears to be critical for provision of ATP necessary for energy demanding processes in the light.

There is also mounting evidence that respiratory ATP production in the light is required to maintain maximum photosynthetic activity (Krömer and Heldt, 1991; Krömer et al., 1993; Krömer, 1995). The link between mitochondrial ATP synthesis and photosynthesis probably occurs via the energy demands of sucrose synthesis: ATP is required for the synthesis of UTP, which in turn is required for UDP-glucose synthesis (Fig. 10). Using the mitochondrial electron transport inhibitor oligomycin, Krömer et al (1993) showed that inhibition of mitochondrial ATP synthesis caused a decline in cytosolic ATP/ADP ratios and a substantial decrease in SPS activity.

Under high irradiance, the inhibition of sucrose synthesis by low ATP supply results in a feedback inhibition of photosynthetic carbon fixation and photosynthetic electron transport (Krömer et al., 1993). Other studies have also demonstrated that limitations in sucrose synthesis can have a feedback inhibitory effect on photosynthesis (Woodrow and Berry, 1988). The degree of inhibition of regulatory enzymes is, however, dependent on the metabolic status of a leaf. For example, at intermediate light intensities, the oligomycin-mediated decrease in mitochondrial and cytosolic ATP/ADP ratios results in a reduced SPS activity, but does not result in a reduced photosynthetic activity (Krömer et al., 1993). At non-saturating irradiance, photon input limits photosynthesis rather than the activities of the regulatory enzymes (Krömer et al., 1993). As a result, limitations in mitochondrial ATP synthesis do not result in feedback inhibition of photosynthetic activity.

In addition to being required for sucrose synthesis, mitochondrial ATP is also likely to be required for sucrose export from source leaf cells. Sucrose export costs account, on average, for 29% of total dark respiration in several starch storing plant species (Bouma et al., 1995).

**H. Role of Mitochondria During Photoinhibition**

Respiration may also help a plant cope with excess photosynthetic redox equivalents (i.e. NADPH). A high NADPH/NADP ratio in the chloroplast can lead through other electron transfer components to the generation of reactive oxygen intermediates (ROI) (Purvis and Shewfelt, 1993; Purvis 1997). A number of studies have indicated that the degree of reduction of the NADP pool in the chloroplast can be decreased by export from the chloroplast via the OAA-malate and/or DHAP-PGA shuttles, followed by oxidation in the mitochondria (Saradadevi and Raghavendra, 1992; Shyam et al., 1993; Hurry et al., 1995; Padmasree and Raghavendra, 1998). It has recently been shown that inhibition of mitochondrial electron transport results in a delay in photosynthetic induction accompanied by increased reduction of chloroplast electron carriers (Igamberdiev et al., 1998). When overreduction of the cell leads to photoinhibition, mitochondrial electron transport can be important also. For example, the increase in photoinhibition in the cyanobacteria *Anacystis nidulans* (Shyam et al.,
following inhibition of mitochondrial respiration, appears to be due in part to the cessation of mitochondrial oxidation of photosynthetic NADPH. The importance of oxidizing excess photosynthetic redox equivalents in the mitochondria may increase at cold temperatures. Low temperatures decrease the demand for NADPH in the chloroplast and increase the potential for photoinhibition (Ball et al., 1991). Increased oxidation of NADPH by respiration would help reduce the potential for severe photoinhibitory damage. There is evidence that this occurs. Firstly, prolonged exposure to low temperatures often results in an increase in respiratory capacity in leaves (Körner and Larcher, 1988). Secondly, it appears that mitochondria of cold-hardened leaves oxidize greater amounts of photosynthetic redox equivalents in the light than non-hardened leaves (Hurry et al., 1995). The latter conclusion was based on the observation that oligomycin, in winter rye, resulted in a greater decrease in maximum photosynthesis in cold-hardened leaves than in non-hardened leaves. This decrease in photosynthesis was not mediated via a decrease in mitochondrial ATP synthesis. Rather, oligomycin inhibited photosynthesis of the cold-hardened leaves by limiting regeneration of RuBP. However, the mechanism behind this effect was not clear.

The pathway of mitochondrial electron transport via which photosynthetic redox equivalents are oxidized will have a large impact on the amount of ATP synthesized in a cell. For example, ATP synthesis via the cytochrome pathway might be beneficial if there is a demand for additional energy in tissues exposed to photoinhibitory conditions (e.g., for UTP generation and/or protein repair). Photoinhibition of Photosystem II results when the rate of damage to D₁ protein exceeds the rate of repair and/or protection (Saradadevi and Raghavendra, 1992; Shyam et al., 1993). Shyam et al. (1993) concluded that mitochondrial ATP is necessary for the repair of the D₁ protein, since inhibition of mitochondrial ATP synthesis by azide and/or the uncoupler FCCP caused an increase in the time needed for recovery from photoinhibition in the cyanobacteria Anacystis nidulans. However, in many species the demand for ATP decreases at low temperatures. Under these conditions it might be more advantageous to oxidize the excess photosynthetic redox equivalents via the non-phosphorylating alternative oxidase (AOX) (Purvis and Shewfelt, 1993). While this hypothesis has not been proven, there is some evidence to support it. The activity of AOX appears to be increased following greening of etiolated leaves (Atkin et al., 1993; Robinson et al., 1995; Ribas-Carbo et al., 1997) and this is probably due to increased protein synthesis (Finnegan et al., 1997). Extended exposure to low temperatures which cause photoinhibition, also increases AOX protein levels (Vanlerberghe and McIntosh, 1992), as does exposure to reactive oxygen intermediates (Wagner, 1995), which can be produced during photooxidation in chloroplasts. Moreover, Prasad et al. (1994) found that chilling leads to an increase in AOX-mediated respiration in maize. Nonetheless, further work is needed before the role of AOX in ameliorating the effects of photoinhibition is confirmed.
IV. Nitrogen Metabolism, Photosynthesis and Respiration

NO$_3^-$ assimilation and amino acid synthesis in leaves requires a large input of ATP, redox equivalents and carbon intermediates. Before NO$_3^-$ can be assimilated into organic nitrogen in leaves, it must be reduced to NO$_2^-$ in the cytosol and then to NH$_4^+$ in the chloroplast (Beavers and Hageman, 1983; Campbell, 1988).

A. Demand for Redox Equivalents by NO$_3^-$ and NO$_2^-$ Reduction

The reduction of NO$_3^-$ to NO$_2^-$ is catalyzed by cytosolic nitrate reductase (NR) and requires NAD(P)H. In leaves, these redox equivalents can be derived from three sources: photosynthesis (export of excess NADPH via the DHAP:3-PGA or OAA-malate shuttles; Mann et al., 1978), glycolysis (oxidation of glyceraldehyde 3-phosphate to 1,3bisphosphoglycerate) and/or mitochondrial respiration (via the OAA-malate shuttle; Shaner and Boyer, 1976).

Expression of a plant to NO$_3^-$ results in a rise in the respiratory quotient (RQ) of some photosynthetic tissues, when measured in darkness (Willis and Yemm, 1955; Bloom et al., 1989). This has led several authors to suggest that mitochondrial redox equivalents are exported to the cytosol and used for NO$_3^-$ reduction (Sawhney et al., 1978; Bloom et al., 1989; Weger and Turpin, 1989). In these tissues, glycolytic NADH supply is insufficient to meet the reductant demands of NO$_3^-$ reduction. It is perhaps not surprising, therefore, that NR activity can be increased in some tissues by the addition of NADH producing metabolites or NADH itself to the NR activity assay media (Klepper et al., 1971; Chanda et al., 1987). However, other studies have reported that reductant supply does not limit NR activity (Tingley et al., 1974; Nicholas et al., 1976; Deane-Drummond et al., 1979; Atkin 1993) or becomes limiting only after a period of extended darkness (Hipkin et al., 1984). Moreover, exposure to NO$_3^-$ does not always result in increased RQ values (Klepper et al., 1971; Deane-Drummond et al., 1979; Hansen, 1980). The degree to which NO$_3^-$ reduction relies on mitochondrial NADH, therefore, varies between species, tissues, and environmental conditions.

An important aspect of the above studies is that RQ was measured in darkness, as was the rate of NO$_3^-$ reduction. In darkness, the NR protein present in leaves is inactivated (Riens and Heldt, 1992; Glaab and Kaiser, 1996), and as such is unlikely to exert a demand for mitochondrial NADH. However, full inactivation can take 10 min or longer to occur; as a result, measurements of gas exchange performed shortly after exposure to darkness will probably be influenced by the redox equivalent demands of NO$_3^-$ reduction.

To our knowledge, no study has investigated the degree to which in situ NO$_3^-$ reduction relies on photosynthetic versus respiratory redox equivalents. Presumably, NO$_3^-$ reduction could rely more on photosynthetic redox equivalents under high light conditions when the NADP pool in the chloroplast is highly reduced. The reliance on photosynthetic and/or mitochondrial redox equivalents, as opposed to glycolytic NADH, will also probably depend on the specific rate of NO$_3^-$ reduction: the demand for non-glycolytic NADH could well be greater when the rate of NO$_3^-$ reduction is high.

NO$_2^-$ produced by NR activity is transported from the cytosol to the chloroplast where it is reduced to NH$_4^+$ by nitrite reductase (NiR) (Lee, 1980). Since this reduction is dependent on ferredoxin, it is light dependent. The rate at which NO$_2^-$ can be reduced to NH$_4^+$ is therefore likely to decrease with decreasing irradiance. Similarly, the reduction of NO$_2^-$ to NH$_4^+$ ceases almost immediately upon exposure to darkness (Riens and Heldt, 1992). NO$_2^-$ reduction to NH$_4^+$ therefore represents a sink for excess photosynthetic redox equivalents. This suggests that the need to export NADPH from the chloroplasts to the respiratory apparatus could be lower in illuminated leaves that are assimilating NO$_3^-$ or NH$_4^+$.

B. Demand for ATP and Carbon Skeletons by NH$_4^+$ Assimilation

In photosynthetic tissues, light-dependent NH$_4^+$ assimilation proceeds in the chloroplast via the coupled activity of ATP-dependent glutamine synthetase (GS) and a F$_{d}$-dependent glutamate synthase (GOGAT), in which glutamate acts as the first recipient of NH$_4^+$ (Miflin and Lea, 1976). NH$_4^+$ is normally only assimilated in leaves following the reduction of NO$_3^-$, as NH$_4^+$ taken up by roots (or produced during root NO$_3^-$ reduction) is not transported through the xylem due to its toxicity. Rather, NH$_4^+$ is assimilated into amino acids before it is transported.

In addition to exerting a demand for ATP and redox equivalents in the chloroplast, NH$_4^+$ assimilation
also requires a continual supply of TCA-cycle derived 2-oxoglutarate (either directly or via citrate). The high rate of amino acid synthesis in the light (Winter et al., 1992) suggests that demand for TCA cycle carbon skeletons is very high. Although the conversion of glutamine to 2 × glutamate requires the input of 2-oxoglutarate, the actual organic acid that is exported from the mitochondria is subject to debate. It has been widely assumed that mitochondria export 2-oxoglutarate, with the concomitant reduction in the release of one molecule of CO₂, NADH and FADH₂. However, more recent evidence suggests that plant mitochondria may export citrate (Hanning and Heldt, 1993), which is converted to 2-oxoglutarate by cytosolic isocitrate dehydrogenase (Chen and Gadal, 1990; Krömer, 1995). ¹³C nuclear magnetic resonance studies using intact leaves have demonstrated that citrate is the major mitochondrial product in the light and that large amounts of citrate accumulate in the vacoule (Gout et al., 1993). If citrate is exported, the TCA cycle will be even more truncated, as two sites of CO₂ and NADH release would be eliminated, in addition to the loss of FAD reduction by succinate dehydrogenase. However, CO₂ would be produced in the cytosol before 2-oxoglutarate could be used for nitrogen assimilation.

C. Requirement for PDC Activity in the Light to Support NH₄⁺ Assimilation

The export of large amounts of citrate to the cytosol in the light requires that there must be continuous carbon input into the TCA cycle. However, previous sections of this chapter highlighted the possibility that light can result in an inactivation of the enzymes controlling substrate input into the TCA cycle (e.g. ME and PDC). How, then, can such high rates of citrate synthesis/export occur in the light? The answer to this question probably lies with the stimulatory effect pyruvate has on PDC activity (Schuller and Randall, 1990). Thus, while exposure to light results in a potential phosphorylation-mediated inhibition of PDC activity by photorespiration, these inhibitory effects can be reduced if sufficient pyruvate is present in the mitochondria. For this to occur, additional carbon must be put into the TCA cycle as OAA, since removal of citrate requires input of a C₄ acid. In such a scheme, OAA/citrate_{out} exchange would occur via the OAA carrier on the mitochondrial inner membrane (Heldt and Flügge, 1987; Hanning et al., 1999). It is possible, therefore, that in the light pyruvate and OAA are the sources of carbon for the mitochondria, while immediately following illumination malate may be the major substrate. It is also worth noting that pyruvate influx to the mitochondria will activate AOX (Millar et al., 1993), while citrate accumulation may stimulate AOX synthesis (Vanlerberghe and McIntosh, 1996).

D. Nitric Oxide Production and the Implications for Respiration

Reduction of NO₃⁻ to NO₂⁻ and then to NH₄⁺ raises the specter of synthesis of intermediary nitrogen oxides that can have major consequences for plant metabolism. Measurements of gaseous emissions from soybean, pea, sugarcane, wheat, corn, sunflower and spinach have demonstrated that NO can be synthesized in plant leaf cells (Klepper, 1979, 1990; Dean and Harper, 1986; Leshem, 1996; Wildt et al. 1997). NO can be produced enzymatically from NO₃⁻ in leaves of soybean and other leguminous species by the constitutive NAD(P)H nitrate reductase (NR) enzyme (nr₄⁻; Dean and Harper, 1988), by non- enzymatic routes (Churchill and Klepper, 1979; Klepper, 1980, 1990), or via putative mammalian-like NO synthases (Sen and Cheema, 1995; Leshem, 1996). Wildt et al. (1997) have shown that NO formation under physiological conditions is positively correlated with the rate of CO₂ fixation in the light, and that substantial NO formation in the dark can occur following a change in the nitrogen regime of plants.

NO formation can provide a signal that the reduction of NO₃⁻ to NH₄⁺ has been partially or fully interrupted. NO acts as a potent inhibitor of cytochrome oxidase with half-maximal inhibition occurring at approximately 25 nM NO, but has a negligible effect on AOX at concentrations of up to 1 μM (Millar and Day, 1996). This differential inhibition may allow AOX, but not cytochrome oxidase, to operate during rapid nitrogen assimilation when the nitrite concentration is high. The significance of this in vivo needs to be addressed.

E. Interaction Between Nitrogen Assimilation and Carbohydrate Metabolism

The above sections have highlighted the large demand nitrogen metabolism has for ATP, redox equivalents and carbon skeletons. To meet these requirements, a plant cell must carefully regulate the flux through the
various metabolic pathways of the chloroplast, cytosol and mitochondria.

The coordination of these pathways has been investigated in detail by Turpin and co-workers using chemostat cultures of green algae. In their experiments, addition of NO$_3^-$ and/or NH$_4^+$ to nitrogen-limited algae results in a rapid, large increase in the demand for carbon skeletons (Elferi and Turpin, 1986; Weger et al., 1988; Elferi et al., 1988) and redox equivalents (Weger and Turpin, 1989). Associated with these demands is a diversion of carbon away from RuBP re-generation (via the export of DHAP) to cytosolic and TCA cycle carbon metabolism (Elferi et al., 1988), resulting in a decrease in net photosynthesis (Elferi and Turpin, 1986) and a concomitant increase in flux through PEP carboxylase (Turpin and Vanlerberghe, 1991), TCA cycle (Elferi and Turpin, 1986) and the respiratory electron transport chain (Weger et al., 1988; Weger et al., 1990). Not surprisingly, respiratory CO$_2$ release and O$_2$ uptake increase following induction of nitrogen assimilation (Weger et al., 1988).

The induction of nitrogen assimilation in green algae relies on carbon input from photosynthetic CO$_2$ fixation via DHAP export from the chloroplast, breakdown of starch/sucrose, and CO$_2$ fixation by PEP carboxylase (Elferi and Turpin, 1986; Turpin and Vanlerberghe, 1991). The importance of CO$_2$ fixation by PEP carboxylase is illustrated by the fact that approximately 0.3 moles of carbon are fixed by PEP carboxylase per mole of nitrogen assimilated in the green alga Selenastrum minutum (Turpin and Vanlerberghe, 1991). The tight regulation of PEP carboxylase activity by carbon substrates (e.g., activity is increased by DHAP, G6P etc) and some nitrogen products (e.g., inhibition by aspartate, glutamate etc), demonstrates that PEP carboxylase activity can be controlled to meet the nitrogen assimilation requirements of plant cells.

PK also increases in activity following induction of NH$_4^+$ assimilation (Turpin et al., 1990). The onset of NH$_4^+$ assimilation in Selenastrum minutum results in a transient removal of adenylate restriction of PK, thus allowing increased flux through glycolysis (Turpin et al., 1990). Moreover, despite adenylate control of PK subsequently being re-established, PK remains active due to the increased concentration of DHAP (a PK activator) and decreased concentration of glutamine (a PK inhibitor) (Turpin et al., 1990).

The increase in TCA cycle activity that occurs when N-limited green algae are exposed to NH$_4^+$ is coupled to increased NADH oxidation by the cytochrome pathway of mitochondrial electron transport (Weger et al., 1988; Weger et al., 1990), despite a large capacity for AOX in these cells (Weger et al., 1990; Weger et al., 1988). The ATP synthesized by the cytochrome pathway is likely to assist in the assimilation of NH$_4^+$ to amino acids.

Work with nitrogen limited wheat leaves also suggests that carbon is re-directed away from sucrose synthesis to meet the carbon requirements of induced nitrogen assimilation: exposure of N-limited wheat leaves to NO$_3^-$ results in a decrease in SPS activity and an increase in PEP carboxylase activity (Van Quy et al., 1991). More work is needed to better understand the situation in higher plants.

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Chapter 8

Regulation of Carbon Fluxes in the Cytosol: Coordination of Sucrose Synthesis, Nitrate Reduction and Organic Acid and Amino Acid Biosynthesis

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Summary

Sugars and amino acids fuel plant growth, development and biomass production. They are required in different amounts and stoichiometries according to developmental stage and environmental challenges and constraints. Extensive interdependence of C and N assimilation operates at several levels, necessitating a complex array of reciprocal controls. Export of amino acids occurs in response to active loading and transport of sucrose in the phloem. Sucrose is the currency of energy exchange between organs and provides ATP for transport processes. Coordination of C and N flow into amino acids and carbohydrates is achieved by regulation of crucial enzyme activities. Nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPC) and sucrose phosphate synthase (SPS) are key enzymes in N assimilation, anaplerotic C flow and sucrose synthesis, respectively. Their activities are coordinated by several mechanisms, the most important of which is protein phosphorylation. Intermediates and products of these pathways regulate gene expression, thereby transmitting information on metabolism to orchestrate C/N flow. A number of putative signal metabolites have been characterized. N metabolites are required for synthesis and activation of enzymes involved in C metabolism (especially for PEPC and SPS). C metabolites are necessary for the synthesis and activation of enzymes catalyzing the assimilation of N (for example, sugars promote NR gene expression). The ‘signals’ involved include nitrate per se, as well as intermediates and products of C and N assimilation such as amino acids, organic acids and sugars.

I. Introduction

Photosynthesis produces assimilatory power in the forms of reduced ferredoxin, NADPH and ATP. These are used to drive photosynthetic carbon assimilation in the chloroplasts which yields sugar phosphates as net product, which can be used for starch formation in the chloroplasts or exported to the cytosol for the synthesis of sucrose (Fig. 1). Sucrose and starch synthesis are tightly coupled to the rate of CO₂ assimilation via the exchange of triose phosphate and inorganic phosphate across the chloroplast envelope membrane (see Chapter 6, Flügge; Foyer and Galtier, 1996).

Carbohydrates are central to the carbon and energy budgets of plants. Sucrose is particularly important since it is a major currency of energy exchange between exporting and importing tissues in higher plants. Starch forms a temporary carbohydrate store in the chloroplasts while sucrose is largely excluded from these organelles being present at the highest concentrations in the cytosol of leaf mesophyll cells. While many plant species predominantly synthesize sucrose and starch, others produce derivatives of sucrose (raffinose saccharides), polymers derived from sucrose (fructans), or acyclic alcohols such as sorbitol or mannitol (see Chapters 12 (Loescher and Everard) and 13 (Cairns et al.); Flora and Madore, 1993; Loescher and Everard, 1996).

In any appreciation of the acquisition of resources for plant growth and development, it is necessary to encompass a wider view, taking into account not only the assimilation of carbon but also the simultaneous assimilation of other essential elements. The most important elements to consider are nitrogen (N) and sulfur (S), as their metabolism is also dependent, either directly or indirectly, on the energy and carbon skeletons provided by photosynthesis. The interdependence between C- and N-metabolism is one major focus of this chapter.

The primary products of photosynthesis in plants and algae are carbohydrates and amino acids. Up to about one third of photosynthetically fixed carbon can be found in amino acids (see Noctor and Foyer, 1998, for review). As well as Gln and Glu which are the products of primary N assimilation, the major amino acids found in plants are: Gly and Ser, which
are largely derived from photorespiration; Ala, which is produced from pyruvate; and Asp and Asn that both arise from oxaloacetate.

Plant growth and productivity is determined not only by photosynthetic capacity, but also by the way in which energy and carbon skeletons are partitioned, allocated and utilized for the assimilation of inorganic elements, particularly nitrogen. Nitrogen can be used in various forms (nitrate, nitrite, ammonium organic nitrogen) by plants. Recently, much attention has been given to N assimilation via absorption of atmospheric and other man-made nitrous pollutants (Rennenberg et al., 1998) but nitrate (taken up by the roots) is considered to be the major N source for higher plants in well aerated soils. Nitrogen uptake mainly occurs across the epidermal and cortical cells of roots. It is subsequently either transported to the vacuole, effluxed out of the cell or transported to other cells. The presence of efficient, membrane-bound transport proteins allows plants and algae to assimilate nitrogen either as nitrate or ammonia (Daniel-Vedele et al., 1998). In many crop species primary N assimilation occurs predominantly in the leaves and is modulated in response to changes in photosynthesis (Kaiser and Forster, 1989; Pace et al., 1990; Ferrario-Méry et al., 1998). In other plants, particularly wild, ruderal species, nitrate is assimilated in roots using carbon skeletons and energy derived from sucrose exported from the leaves to the roots.

A. The Provision of Energy and Carbon Skeletons

N assimilation involves two processes: (1) the oxidative formation of oxoacids from triose phosphate, and (2) the reductive incorporation of ammonia (derived from nitrate) into oxoacids to form amino acids (equations 1–4). Nitrate assimilation leads firstly to the formation of ammonium (Eqs. (1 and 2)).

Cytosol:
\[
\begin{align*}
\text{NO}_3^- + \text{NADH} + \text{H}^+ & \xrightarrow{\text{nitrate reductase}} \text{NO}_2^- \\
+ \text{NAD} + \text{H}_2\text{O} & \xrightarrow{} 
\end{align*}
\] (1)

Stroma:
\[
\text{NO}_2^- + 6\text{Fd}_{\text{red}} \xrightarrow{\text{nitrate reductase}} \text{NH}_3^- + 6\text{Fd}_{\text{ox}}
\] (2)

The NR isoforms involved in primary N assimilation are cytosolic proteins that use NADH or NADPH to reduce nitrate to nitrite Eq. (1). In addition, succinate-oxidizing nitrate reductase (PM-NR) isoforms are localized on the plasmamembrane of roots (Stöhr and Ullrich, 1997) and in algae (Stöhr et al., 1993). It is possible that the membrane-bound form of the enzyme may also function to produce nitric oxide (NO) from nitrite. NO is a powerful regulator of cytochrome c oxidase in plant mitochondria (Chapter 7 (Atkin et al.); Millar and Day, 1996). There is mounting evidence that NO is a byproduct of primary N assimilation in plants, produced when nitrite accumulates (Millar and Day, 1997). The PM-NR isoforms may, therefore, have a role in signal transduction in situations where nitrate reduction exceeds the rate of ammonium formation.

In higher plants, ammonia is assimilated via the chloroplastic enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). The reaction catalyzed by GS Eq. (3) is considered to be the major route facilitating the incorporation of inorganic nitrogen into organic molecules, in conjunction with glutamate synthase Eq. (4), which recycles glutamate and incorporates carbon skeletons into the cycle for the transfer of amino groups (Lea and Miflin, 1974; Coschigano et al., 1998).
The acceptor for the amino groups in the reaction catalyzed by GOGAT is 2-oxoglutarate (2-OG), but a wide range of other organic acids act as the precursors for the synthesis of other amino acids. The shortest metabolic route for generation of 2-OG and other organic acids from triose phosphate is through glycolytic metabolism of triose phosphate and partial anaplerotic tricarboxylic acid (TCA) cycle activity (Fig. 2). It is now widely accepted that respiratory carbon flow must occur in the light to produce metabolic precursors (Noctor and Foyer, 1998). Oxidative phosphorylation is also considered to be at least partially operative in the light and necessary for sucrose synthesis (Krömer et al., 1988; Krömer and Heldt, 1991; Krömer, 1995). Carbon metabolism associated with 2-OG formation not only provides carbon skeletons for the assimilation of ammonium but also organic acids for acid-base regulation and is a source of ATP (Chapter 7, Atkin et al.). The respiratory carbon flow required for photosynthetic N assimilation has the potential to form significant amounts of ATP and may be able to contribute ATP to meet the high ATP/reductant requirements of photosynthetic C assimilation (see Chapter 7, Atkin et al.; Noctor and Foyer, 1998).

While the major route of 2-OG production involves partial operation of the TCA cycle in the mitochondrion, 2-OG synthesis may also occur via a cytosolic isocitrate dehydrogenase (Fig. 2). In the TCA cycle, acetate units are oxidized to and cannot, therefore, lead to net synthesis of cycle metabolites. Removal of any of the intermediates for biosynthetic reactions will require replenishment of carbon skeletons subsequent to the steps involving decarboxylation (i.e. as \( C_4 \) acids). These could be supplied as succinate, fumarate, malate or oxaloacetate. In mature leaves, the most important input of \( C_4 \) acids for 2-OG synthesis is thought to be as oxaloacetate, generated by the enzyme phosphoenolpyruvate (PEP) carboxylase (PEPC). Instead of all of the PEP being converted to pyruvate for oxidative decarboxylation prior to entry into the cycle, approximately half of the PEP is carboxylated to oxaloacetate via PEPC, thus allowing assimilatory 2-OG production through reactions catalyzed by citrate synthase, aconitase and isocitrate dehydrogenase (Fig. 2). The net reaction can be represented simply as:

\[
2 \text{PEP} + \text{NAD}^+ \rightarrow 2 \text{OG} + \text{CO}_2 + 2 \text{Pi} + \text{NADH} \tag{5}
\]

PEP carboxylase therefore has a central function in the C and N economy of the leaf. Different PEPC isoforms perform essential roles in photosynthesis in \( C_4 \) and CAM plants (Duff and Chollet, 1995) and in anaplerotic metabolism in all plants (O’Leary, 1982). The PEPC isoforms differ in their physiological and biochemical properties but all of the isoforms are regulated by transcriptional and post-translational (protein phosphorylation) controls (Tagu et al., 1991; Cushman et al., 1993; Pacquit et al., 1993; Hartwell et al., 1996). Phosphorylation of the PEPC protein activates the enzyme leading to decreased inhibition by malate or increased activation by G6P. The \( K_m \) and \( V_{\text{max}} \) values measured for the photosynthetic PEPC isoforms in \( C_4 \) plants are higher than those reported for \( C_3 \) isoforms (Jiao and Chollet, 1991; Duff and Chollet, 1995).

Gln acts as an amino donor in relatively few reactions but amino groups are transferred from Glu to oxaloacetate and pyruvate to produce Asp and Ala, respectively (see Ireland, 1997, for review). In photorespiration, amino groups are transferred to glyoxylate to produce Gly and, subsequently, Ser. PEP and erythrose-4-P are the C skeletons used for the synthesis of aromatic amino acids (Phe, Trp, Tyr). Aspartate is the starting-point for the synthesis of Asn, Thr, Lys and Met (Ireland, 1997). C skeletons for the branched-chain amino acids come from pyruvate (Val, Leu) or 2-oxobutyrate (11e) and acetyl CoA. Ribose-5-P is used for His synthesis and 2-OG is required for Arg synthesis. In almost all of these pathways the immediate amino donor is Glu or Asp (Heldt, 1997).

**B. Effects of Nitrate Assimilation on Photosynthesis, Sucrose and Starch Synthesis**

While the relative composition of the photosynthetic apparatus is largely unaffected by N supply (Evans, 1983; Evans and Terashima, 1987), the availability of nitrogeplays a crucial role in determining overall photosynthetic capacity (Evans and Terashima, 1987; Heckathorn et al., 1997), since about three-quarters of the N in leaves is contained in chloroplast proteins.
The availability of N also affects leaf growth (Makino et al., 1984), shoot to root ratios (Scheible et al., 1997a) and the capacity of plants to recover from drought (Heckathorn et al., 1997; Ferrario-Méry et al., 1998). The assimilation of N in leaves leads to a drop in the assimilatory quotient (net CO₂ fixed per net O₂ evolved). This is due to a stimulation of photosynthetic O₂ evolution as a result of nitrite reduction (de la Torre, 1991) but concurrent respiratory O₂ uptake may partially offset this increase. Unregulated competition for reducing power and ATP between the processes of carbon and nitrogen assimilation is not observed in the leaves of higher plants. Nitrate assimilation does not inhibit CO₂ assimilation in leaves but it changes the partitioning of carbon, decreasing the rates of sucrose and starch synthesis and stimulating the flow of carbon through the pathways of organic acid biosynthesis. This coordination is achieved by a network of controls that regulate the activities of enzymes regulating the flux through these pathways.

Appropriate provision of triose phosphate in the required stoichiometries for sucrose synthesis and for the formation of oxoacids for primary N assimilation into amino acids requires precise coordination between the pathways of C and N assimilation in plants. Many lines of evidence point to numerous steps of reciprocal control at the levels of gene transcription, mRNA stability and translation, as well as post-transcriptional regulation. Increased nitrate assimilation, for example, leads to increased synthesis of organic acids (Kaiser and Forster, 1989; Foyer and Ferrario, 1994), and decreased sucrose and starch synthesis (Waring et al., 1985; Fichtner and Schulze, 1992). Glutamine, glutamate, aspartate, 2-oxoglutarate and malate accumulation are modified following addition of either nitrate or ammonium to the alga Selenastrum minutum and carbon flow into organic acids is stimulated by coordinated activation of pyruvate kinase and PEPC (Huppe and Turpin, 1994; Turpin et al., 1997). In higher plants, there is
evidence for allosteric regulation of these enzymes in response to N supply (Champigny and Foyer, 1992; Turpin et al., 1997). Transgenic plants lacking the primary enzyme of CO₂ fixation, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), have low NR activity and accumulate more nitrate but contain less glutamine and other amino acids than untransformed controls (Stitt and Schulze, 1994). Such studies indicate that low rates of photosynthesis are accompanied by low rates of nitrate assimilation and vice versa.

The activities of several enzymes in the cytosol, including NR, SPS and PEPC, are modulated in response to changes in photosynthesis. In the light, photosynthesis becomes active and signals are transmitted from the chloroplast to the cytosol so that NR, SPS and PEPC are activated within a few minutes (see Section II). NR and SPS are activated by dephosphorylation and PEPC is activated by phosphorylation. The activation of the enzymes increases their capacities and modulates flux through sucrose synthesis, nitrate assimilation and organic acid synthesis (Champigny and Foyer, 1992). Coordinate regulation of PEPC (Foyer et al., 1994; Duff and Chollet, 1995; Li et al., 1996) and SPS (Champigny et al., 1992; Huber and Huber, 1996) has been suggested to be achieved via regulation by protein phosphorylation (see Section II). SPS, NR and PEPC are not always regulated in parallel, however, indicating that the metabolic and environmental factors that modulate the activities of these enzymes are not strictly identical.

In higher plants and in algae N assimilation stimulates respiratory metabolism in both the light and dark thereby directing carbon from starch and sucrose biosynthesis to respiration and amino acid biosynthesis. This activation of respiration results in inhibition of photosynthesis in algae, but not in higher plants. This is caused by redirection of carbon and reducing power to capture limited resources. The assimilation of NO₃⁻ in both light and dark activates the oxidative pentose phosphate pathway providing reducing power for nitrate reduction and C skeletons for amino acid synthesis (Vanlerberghe, 1991; Huppe et al., 1992). In algal systems glucose 6-phosphate dehydrogenase (G6PDH), which catalyzes the first step of this pathway and is regulated by reversible oxidation/reduction by the ferredoxin-thioredoxin system, is modified in the presence of nitrate. At limiting NO₃⁻ light-dependent reduction of chloroplastic G6PDH inhibits the oxidative pentose phosphate pathway during photosynthetic carbon assimilation. The addition of nitrate causes rapid oxidation and activation of G6PDH in algae, stimulating carbon flow through the oxidative pentose phosphate pathway (Huppe et al., 1994). In contrast, the assimilation of ammonium activates glycolysis and the TCA cycle but not G6PDH in algae (Huppe et al., 1992).

C. Effects of Sucrose on Nitrogen Assimilation

Sucrose and 2-OG have been shown to be signal compounds that communicate and integrate information on metabolic status to ensure the appropriate balance of supply and demand between source and sink reactions. Sucrose and other signals arising from primary carbon metabolism directly influence photoassimilate production and utilization through the control of gene expression. Current concepts implicate hexokinase activity in sugar sensing (Chapter 10, Graham and Martin; Jang et al., 1997) but other components at the plasmamembrane such as two sugar sensors Snf3p and Rgt2p are also involved (Ozcan et al., 1996; Smeekens and Rook, 1997).

Signals from carbon metabolism regulate nitrate assimilation and amino acid synthesis in response to changes in the availability of carbohydrate (Morcuende et al., 1998). The abundance of transcripts for several enzymes involved in nitrogen metabolism is modified by sugars. For example, feeding sucrose and glucose to leaves increased NR transcript abundance (Cheng et al., 1992; Vincentz et al., 1993) and decreased the asparagine synthetase transcript abundance (Lam et al., 1994, 1996). Sugars also lead to increased activation of NR (Kaiser and Spill, 1991; Kaiser and Huber, 1994). Sucrose acts in concert with nitrate (and antagonistically to Gln) to increase NR activity (Morcuende et al., 1998). Although sugars contribute to the increase of nia transcript, NR protein, and post-transcriptional activation of NR after illumination of mature leaves, the abundance of nia transcripts and NR protein have been found to decrease as the photoperiod progresses, even though carbohydrates continue to accumulate in the leaf (Galangau et al., 1988; Scheible et al., 1997b). In tobacco, the abundance of NR gene (nia) transcripts is maximal at dawn, decreasing throughout the day, whereas in maize, NR transcripts are maximal 1 to 2 h into the photoperiod (Huber et al., 1994). These changes probably reflect the complex
interplay of modulation by nitrate (Cheng et al., 1986), sucrose (Cheng et al., 1992; Vincentz et al., 1993) and Gln (Vaucheret et al., 1990; Deng et al., 1991; Vincentz et al., 1993; Hoff et al., 1994; Dzuibany et al., 1998) which stimulate and inhibit transcription of *nia*. Dzuibany et al. (1998) have argued against a role for Gln as an effective repressor of *nia2* transcript accumulation in *Arabidopsis*. Recent studies with transformed tobacco expressing an antisense Fd-GOGAT construct suggest that 2-oxoglutarate acts antagonistically to Gln, hence Gln will only inhibit NR transcription when 2-oxoglutarate is low (see Section V). Studies of transgenic tobacco plants overexpressing *nia* under the control of a constitutive promoter indicate that the decay is due to post-transcriptional regulation (Vincentz and Caboche, 1991; Ferrario et al., 1995) and not just a consequence of decreased *nia* transcription rate. Sucrose and glucose act antagonistically on NR activation (Kaiser and Huber, 1994; Scheible et al., 1997b).

Nitrate and sugars modulate translation of NR mRNA and affect the stability of NR protein. NR protein content and NR activity are generally higher in the light than in the dark providing strong evidence that post-transcriptional controls are important; neither correlate with transcript abundance (Scheible et al., 1997b). Studies of transgenic plants overexpressing a modified NR that is not susceptible to phosphorylation, together with observations on NR phosphorylation state and NR stability (Kaiser and Huber, 1997), indicate that phosphorylation regulates NR degradation as well as NR activity. Increased expression of PEPC, cytosolic pyruvate kinase, citrate synthase and the NADP-dependent isocitrate dehydrogenase has been observed following addition of nitrate to N-deficient plants (Scheible et al., 1997c). This is considered necessary to allow increased synthesis of organic acids, while decreased ADP-glucose pyrophosphorylase activity inhibits starch synthesis in these conditions (Scheible et al., 1997c).

**D. Covalent Modification and Allosteric Control**

Protein phosphorylation is generally recognized as being an important process controlling many aspects of signal transduction, development and membrane transport, but in the area of metabolism, there are still relatively few enzymes known to be regulated by this mechanism. Most of the currently recognized enzymes are localized in the plant cell cytoplasm, where they are involved in major pathways of C- and N-metabolism. The relevant enzymes, and the pathways in which they function, are shown in simplified fashion in Fig. 3. With many of the enzymes, their phosphorylation status (and hence enzymatic activity) is modulated in leaves by light/dark transitions. Enzymes controlled in this manner include SPS, PEPC, and NR (for reviews see Huber et al., 1994; Huber and Kaiser 1996; Chollet et al., 1996). In addition, recent evidence suggests that the activity of the plasma membrane H+-ATPase may also be regulated by phosphorylation in a complex process that also involves 14-3-3 proteins (Moorhead et al., 1996).

**E. Analysis of C/N Metabolism in Mutants and Transformed Plants**

Transformed plants and mutants have been widely used to study the pathways of carbon, nitrogen and ammonium assimilation in plants. While it is not possible to provide an exhaustive list of the results of such studies to date, Table 1 attempts to summarize the major findings of key studies involving SPS, PEPC, NR, GS and GOGAT. Such studies have yielded interesting and occasionally surprising results. Early studies with mutants deficient in NR suggested that this enzyme was present in vast excess of that required to support growth and development. Protein content and growth were not decreased in barley mutants with only 10% of NR activity measured in the wild type controls (Warner and Kleinhofs, 1981; Warner and Huffaker, 1989). Similar results were obtained in *Arabidopsis* mutants (Wilkinson and Crawford, 1993). *Nicotiana plumbaginifolia* and *N. tabacum* mutants with 40–50% of the wild type NR activity were also unaltered in phenotype to the wild-type controls (Müller and Mendel, 1989; Vaucheret et al., 1990; Vincentz and Caboche 1991; Dorbe et al., 1992; Scheible et al., 1997a). Only when the evidence for post-transcriptional and post-translational regulation of NR was presented was this situation clarified. Regulated changes in the activation state of the enzyme may be used to compensate for decreases in maximal extractable enzyme activity, to maintain the appropriate balance between C and N acquisition in the plant (Scheible et al., 1997b). Changes in NR expression together with regulation of NR activity, allowing assimilation to occur for a longer period each day, were found to offset the effects of decreased N assimilation capacity (Scheible...
et al., 1997b). Transformed plants with decreased NR activity have also been used to demonstrate a role for nitrate in the regulation of root-shoot allocation (Scheible et al., 1997a) and in the modulation of carbon partitioning (Scheible et al., 1997c).

II. Sucrose-P Synthase

High SPS activities are found in leaves where they are subject to complex regulatory controls involving: i) molecular genetic regulation of gene expression and steady state enzyme protein contents, ii) allosteric regulation involving Glc-6-P activation and Pi inhibition, and iii) protein phosphorylation. Many different mechanisms work in parallel to adjust SPS protein content as well as the catalytic activity of the enzyme protein. Phosphorylation of SPS was originally characterized as the mechanism underlying light/dark modulation of SPS activity (Huber and Huber, 1992). More recently phosphorylation of SPS
### Table 1. A summary of some of the results obtained in various studies on mutants and transformed plants modified in nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPC), glutamine synthetase (GS) and glutamate synthase (GOGAT)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of mutation or transformation</th>
<th>Plant material</th>
<th>Consequences on C, N metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheible et al., 1997a</td>
<td>mutant deficient for 1 or 2 nia gene</td>
<td><em>N. tabacum</em></td>
<td>Modification in diurnal regulation of NR mRNA transcription post-translational modification and turnover of NR</td>
</tr>
<tr>
<td>Vaucheret et al., 1990</td>
<td>tobacco nia2 gene</td>
<td><em>N. plumbaginifolia</em> mutant deficient in NR</td>
<td>Functional complementation of NR but low level of NR activity</td>
</tr>
<tr>
<td>Vincentz and Caboche, 1991</td>
<td>35S nia2</td>
<td><em>N. plumbaginifolia</em> mutant deficient in NR</td>
<td>Increase in NR activity and mRNA</td>
</tr>
<tr>
<td>Dorbe et al., 1992</td>
<td>tomato nia gene</td>
<td><em>N. plumbaginifolia</em> mutant deficient in NR</td>
<td>NR activity restored and regulated but low level of NR</td>
</tr>
</tbody>
</table>
| Foyer et al., 1993 | a) 35S nia2  
                             b) tobacco nia2 gene | *N. plumbaginifolia* mutant deficient in NR | a) Increase in NR activity; increase in amino acid; no change in starch and soluble sugar  
                             b) Low NR activity; decrease in leaf protein, Chl, amino acid; increase in starch and sucrose |
| Foyer et al., 1994 | 35S nia2 or tobacco nia2 gene | *N. plumbaginifolia* mutant deficient in NR | Maximum rate of photosynthesis modified in low NR transformant; unchanged in high NR expressor |
| Quilleré et al., 1994 | 35S nia | *N. plumbaginifolia* mutant deficient in NR | Increase in NR activity; decrease in NO$_3$ content; increase in Gln and malate contents |
| Nussaume et al., 1995 | 35S deleted nia2 | *N. plumbaginifolia* mutant deficient in NR | Suppression of post-transcriptional regulation of NR by light increase in Gln and Asn contents |
| Ferrario et al., 1995 | 35S nia2 | *N. plumbaginifolia* mutant deficient in NR | NR activity photosynthesis and biomass decreased by low nitrogen supply |
| Ferrario et al., 1996 | 35S nia2 | *N. plumbaginifolia* mutant deficient in NR | NR activation state is not influenced by NO$_3$ |
| Navarro et al., 1996 | 35S nia2 | *Chlamydomonas* | Inactivation of NR in absence of NO$_3$ |
| Ferrario-Méry et al., 1997 | 35S nia2 | *N. plumbaginifolia* mutant deficient in NR | Increase in NR activity; increase in Gln level and high C/N ratio maintained in high CO$_2$ conditions |
| Ferrario-Méry et al., 1998 | 35S nia2 | *N. plumbaginifolia* mutant deficient in NR | Delay in the decrease in NR activity due to drought |
| Scheible et al., 1997b | a) mutant deficient for 1 or 2 gene  
                             b) tobacco nia2 gene | a) *N. tabacum*  
                             b) *N. tabacum* mutant deficient in NR | Varying levels of NR accumulation in NO$_3$ but low amino acid and protein contents and increased shoot to root allocation correlated to a decreased sugar allocation to roots |
<p>| Scheible et al., 1997c | tobacco nia2 gene | <em>N. tabacum</em> mutant deficient in NR | Varying levels of NR accumulation of, NO$_3$ and induction of NR, NiR, GS1 GOGAT, PEPC, ICDH and repression of ADPGlc PPase |</p>
<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of mutation or transformation</th>
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<th>Consequences on C, N metabolism</th>
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<tr>
<td><strong>PEPC</strong></td>
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<tr>
<td>Tagu et al., 1991</td>
<td>C₄ Sorghum PEPC</td>
<td>N. tabacum</td>
<td>Expression in leaves but not in roots</td>
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<tr>
<td>Hudspeth et al., 1992</td>
<td>a) Cab C₄ maize PEPC</td>
<td>N. tabacum</td>
<td>a) Expression in mesophyll cells; increase in PEPC activity and malate content; no change in photosynthesis</td>
</tr>
<tr>
<td></td>
<td>b) C₄ maize PEPC</td>
<td></td>
<td>b) Aberrant transcription initiation; light regulation maintained</td>
</tr>
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<td>Cushman et al., 1993</td>
<td>CAM <em>Mesembryanthemum</em> PEPC promoter-GUS</td>
<td>N. tabacum</td>
<td>Role of 5’ untranslated region in salt stress inducibility</td>
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<td>Matsuoka et al., 1994</td>
<td>C₄ maize promoter-GUS</td>
<td>rice</td>
<td>Light regulation maintained</td>
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<td>Kogami et al., 1994</td>
<td>35S c₄ maize PEPC</td>
<td>N. tabacum</td>
<td>Increase in PEPC; decrease in growth rate and Chl content</td>
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<td>Stokhous et al., 1994</td>
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<td>N. tabacum</td>
<td>Role of 5’ untranslated region in C₄ specific expression</td>
</tr>
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<td>Gehlen et al., 1996</td>
<td>a) bacterial PEPC</td>
<td>potato</td>
<td>a) Decrease in growth rate; increase in malate; no change in photosynthesis</td>
</tr>
<tr>
<td></td>
<td>b) antisense bacterial PEPC</td>
<td></td>
<td>b) No change in photosynthesis</td>
</tr>
<tr>
<td>Pathirana et al., 1997</td>
<td>nodule alfalfa PEPC promoter-GUS</td>
<td>alfalfa</td>
<td>Role of 5’ untranslated region in nodule specific expression</td>
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<td><strong>GS/GOGAT</strong></td>
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<td>Eckes et al., 1989</td>
<td>35S alfalfa GS1</td>
<td>N. tabacum</td>
<td>Increase in GS, decrease in NH₄⁺; no change in amino acid composition</td>
</tr>
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<td>Hrel et al., 1992</td>
<td>35S soybean GS1</td>
<td>N. tabacum</td>
<td>Increase in GS; induction of mRNA for a native cytosolic GS</td>
</tr>
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<td>Temple et al., 1993</td>
<td>35S alfalfa GS</td>
<td>N. tabacum</td>
<td>a) Increase in GS1</td>
</tr>
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<td>a) sense construct</td>
<td></td>
<td>b) Decrease in GS1 and GS2; decreases in PEPC and hydroxyxypyruvate reductase</td>
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<tr>
<td></td>
<td>b) antisense construct</td>
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<td>Low viability</td>
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<tr>
<td>Temple et al., 1994</td>
<td>phloem specific promoter-antisense GS1</td>
<td>alfalfa</td>
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<tr>
<td>Vincentz et al., 1997</td>
<td>35S soybean GS1</td>
<td><em>Lotus corniculatus</em></td>
<td>Increase in GS; increase in aa, NH₄⁺ due to early senescence; decrease in carbohydrates in roots</td>
</tr>
<tr>
<td>Temple and Sengupta-Gopalan, 1997</td>
<td>nodule or vascular specific promoter-alfalfa GS1 (differing in 3’ untranslated region)</td>
<td>alfalfa</td>
<td>Decrease in GS in nodule; increase in GS in stem</td>
</tr>
</tbody>
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Chapter 8 Regulation of Carbon Fluxes in the Cytosol

<table>
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<th>Reference</th>
<th>Type</th>
<th>Species</th>
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<tr>
<td>Somerville et al., 1980</td>
<td>Fd-GOGAT deficient mutant</td>
<td>Arabidopsis</td>
<td>a) In high CO₂; no changes in Rubisco, GS, GDH and in photosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) In air; increase in NH₄⁺, Glu, organic acids; decreases in Glu and amino acid; decrease in photosynthesis</td>
</tr>
<tr>
<td>Kendall et al., 1986</td>
<td>Fd-GOGAT deficient mutant</td>
<td>barley</td>
<td>a) In high CO₂; no change in photosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) In air; increase in malate, Glu, Asn and NH₄⁺; decreases in Asp, Ala, Glu, Gly and Ser; decrease in photosynthesis; ¹⁴C incorporation increases into sugars P, glycollate and malate and decreased into Ser, Gly, glyceraldehyde and sucrose</td>
</tr>
<tr>
<td>Blackwell et al., 1988a</td>
<td>Fd-GOGAT and/or GS deficient mutant</td>
<td>barley</td>
<td>Increase in NH₄⁺ in air; decrease in photosynthesis in air; reduced ability to incorporate ¹⁴C derived from ¹⁴CO₂ into sucrose in air</td>
</tr>
<tr>
<td>Blackwell et al., 1987</td>
<td>GS2 deficient mutant</td>
<td>barley</td>
<td>Viable only in non-photorespiratory conditions; increase in NH₄⁺ in air; inhibition in photosynthesis in air</td>
</tr>
<tr>
<td>Wallsgrove et al., 1987</td>
<td></td>
<td></td>
<td>In air; accumulation of NH₄⁺; inhibition of photosynthesis is probably due to the depletion of amino donors for glyoxalate</td>
</tr>
<tr>
<td>Matson et al., 1997</td>
<td></td>
<td></td>
<td>The two Fd-GOGAT genes have distinct roles in photorespiration and primary nitrogen assimilation</td>
</tr>
<tr>
<td>Morris et al., 1989</td>
<td>Fd-GOGAT deficient mutant</td>
<td>Arabidopsis</td>
<td>a) In high CO₂; organic nitrogen exported (Gln) from roots to shoots in the xylem sap is reduced in GS deficient mutants and enhanced in the Fd-GOGAT mutant (¹⁵N feeding experiments); ¹⁵N supplied to excised leaves is accumulated in Gln but at very low level in other amino acid in Fd-GOGAT deficient mutants</td>
</tr>
<tr>
<td>Coschigano et al., 1998</td>
<td>Fd-GOGAT deficient mutant</td>
<td>Arabidopsis</td>
<td>Growth is not severely impaired in air</td>
</tr>
<tr>
<td>Joy et al., 1992</td>
<td>Fd-GOGAT or GS deficient mutant</td>
<td>barley</td>
<td>a) In air; no change in Rubisco, prot Chl ammonium increase; amino acid decrease and increase in Ser/Gly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) In air; decrease in Rubisco, prot, Chl, NO₃; no change in ammonium and amino acid but Gln increase Glu and ASP decrease Ser/Gly increase</td>
</tr>
<tr>
<td>Hausler et al., 1994a</td>
<td>Fd-GOGAT with reduced activities (63–75%)</td>
<td>barley</td>
<td>a) In air; electron requirement per CO₂ assimilated is decreased</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) In air; CO₂ assimilation is reduced</td>
</tr>
<tr>
<td>Hausler et al., 1994b</td>
<td>Fd-GOGAT with reduced activities (63–75%)</td>
<td>barley</td>
<td>a) In air; serine-glyoxylate aminotransferase decreased</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glutamate and alanine: glyoxylate aminotransferase increased</td>
</tr>
<tr>
<td>Hausler et al., 1996</td>
<td>Fd-GOGAT with reduced activities (63–75%)</td>
<td>barley</td>
<td>Negative correlation between the activation state of Rubisco and glyoxylate content</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) GS mutants with reduced activities (47–97%)</td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Type of mutation or transformation</td>
<td>Plant material</td>
<td>Consequences on C, N metabolism</td>
</tr>
<tr>
<td>-------------------------</td>
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</tr>
</tbody>
</table>
| Lea et al., 1992        | Fd-GOGAT deficient mutant          | pea            | a) In air; increase in Gln; decrease in Asn and other amino acid  
b) In high CO$_2$ or in air; $^{15}$N Gln feeding experiments leads to very low $^{15}$N incorporation in other amino acids |
| Edwards et al., 1990    | pea chloroplast GS2 promoter-GUS   | *N. tabacum*   | Cell specific expression of GS2 in leaves and GS3A in phloem |
|                         | pea cytosolic GS3A promoter-GUS    |                |                                 |
| Miao et al., 1991       | soybean cytosolic GS15 promoter-GUS| *Lotus corniculatus*  
* N. tabacum*        | Root specific expression inducibility by NH$_4^+$ in *Lotus* but not in tobacco roots |
| Brears et al., 1991     | a) pea cytosolic GS3A promoter-GUS | *N. tabacum*    | Expression in phloem and in root nodule not altered; identification of a nuclear DNA binding protein |
|                         | b) 5' deleted GS3A promoter-GUS    | alfalfa        |                                 |
| Cock et al., 1992       | bean plastidic GS2 5' deleted     | *N. tabacum*   | Tissue specific and light regulation of GS2 related to an essential region for promoter |
|                         | promoter-GUS                       |                |                                 |
| Tjaden and Coruzzi, 1994| pea chloroplastic GS2-AT rich      |                | Decrease in GUS activity and isolation of a novel DNA binding protein |
|                         | clement deleted promoter-GUS       |                |                                 |
has also been implicated in the activation of the enzyme that occurs when leaf tissue is subjected to osmotic stress (Toroser and Huber, 1997).

A. Regulatory Phosphorylation Sites

SPS is phosphorylated on multiple seryl residues, only one of which is involved in the dark-inactivation of the enzyme (Huber and Huber, 1992). The phosphorylated residue was subsequently shown to be Serine-158, and this residue is conserved among species (Huber and Huber, 1996). Phosphorylation of Ser-158 reduces SPS activity by altering affinities for substrates and effectors, without affecting maximum catalytic activity. Recent evidence suggests that there is a second regulatory phosphorylation site at Ser-424, which is phosphorylated when leaf tissue is subjected to osmotic stress (Toroser and Huber, 1997). This site is also conserved among species. Phosphorylation of Ser-424 may activate the enzyme by antagonizing the inhibitory effect of Ser-158 phosphorylation, and thereby allow sucrose synthesis to occur when it would otherwise be restricted.

B. Sucrose-P Synthase-Kinase

Ion-exchange chromatography of spinach leaf proteins resolves several peaks of protein kinase activity that will phosphorylate a synthetic peptide based on the amino acid sequence surrounding Ser-158 (McMichael et al., 1995; Douglas et al., 1996). However, not all of the kinases that will phosphorylate the synthetic peptide are capable of phosphorylating the regulatory site in the native protein. One of the major protein kinase activities that phosphorylates Ser-158 in the native protein has been designated Peak III (or PK_{III}) and tends to co-purify with SPS. PK_{III} is Ca^{2+}-independent, has a native Mr of ~150,000 (McMichael et al., 1995), and a catalytic subunit of ~ 60 kDa that is recognized by antibodies against RKIN1, a plant SNF1 homolog (Douglas et al., 1996). The SNF1 family of protein kinases was named for the sucrose non-fermenting kinase of yeast, and subsequently, kinases with similar catalytic sub-units were identified in mammalian and plant systems (Halford and Hardie, 1998). A characteristic property of the SNF1-like kinases is that they are often regulated by phosphorylation, and this has been demonstrated for PK_{III} (Douglas et al., 1996). Dephosphorylation/inactivation of PK_{III} is catalyzed by PP2C in vitro but details of the phosphorylation/activation reaction are lacking. If a distinct kinase is involved, then a phosphorylation cascade would be involved that could be subject to regulation. It is not known at present whether the phosphorylation state, and hence activity, of PK_{III} varies in vivo. If so, this could also contribute directly to the control of SPS activation state.

One factor that is thought to regulate the PK_{III}-catalyzed phosphorylation/inactivation of SPS are metabolites, in particular Glc-6-P (Weiner et al., 1992). The Glc-6-P could act by binding to SPS at its allosteric site, or by directly affecting the protein kinase (PK_{III}). The limited evidence to date suggests that direct effects on the kinase may be involved, although some effect of binding to SPS as well may also contribute (McMichael et al., 1995). Regulation of PK_{III} by Glc-6-P could involve one of the putative regulatory subunits thought to associate with the ~60-kDa catalytic subunit to produce the native oligomeric protein.

C. Sucrose-P Synthase-Protein Phosphatase

Phospho-Ser-158-SPS can be dephosphorylated and activated in vitro by the catalytic subunit of protein phosphatase (PP) PP2A and not PP1 (Weiner, 1997). However, in vivo PP2As likely occur as oligomeric proteins, with the ~35-kDa catalytic subunit associated with one or more regulatory sub-units. One of the interesting regulatory properties of the PP2As that act on SPS is inhibition by Pi. Interestingly, the activity and regulatory properties of SPS-PP2A appear to change with light/dark treatment of leaves; the enzyme from illuminated leaves has a slightly higher activity and several-fold reduced sensitivity to Pi inhibition. The molecular basis for the shift in properties is not known, but involves cytoplasmic protein synthesis as the light-activation of SPS can be inhibited by cycloheximide (Weiner et al., 1992), and interconversion of the native enzyme between a dimeric and trimeric form (Weiner, 1997).

The action of PP2As on SPS may also be influenced by amino acids. It has been observed that the light activation of SPS in detached leaves can be reduced by feeding amino acids via the transpiration stream (Huber et al., 1996). The effect was not specific for a given amino acid, suggesting that it may be a rather general response. Inhibition of SPS-PP2A activity by amino acids has been observed in vitro, but relatively high concentrations (10 to 50 mM) were required which raises the possibility that the effect
may not be of physiological significance. However, amino acids are found at relatively high concentrations in the cytosol of leaf cells (Reins et al., 1994). Interestingly, changes in tissue amino acid content often parallel changes in SPS activation state, as would be expected if they inhibit the requisite PP2A(s). For example, in spinach leaves, accumulation of amino acids during the photoperiod tends to parallel the progressive inactivation of SPS that occurs. Another example involves *Vicia faba* seed development. Cotyledon SPS is partially inactivated (presumably by phosphorylation) during the storage phase (40 to 50 days after fertilization) and this generally corresponded with an accumulation of free amino acids (Weber et al., 1996). Furthermore, in vitro culture of *V. faba* cotyledons in a high hexose medium increased the activation state of SPS and decreased the level of free amino acids compared to cultures on a high sucrose medium. Thus, accumulation of free amino acids (presumably in excess of some optimum level) has been correlated with inactivation of SPS in several different tissues. Inhibition of SPS-PP2As by amino acids could be responsible.

### D. Manipulation of Sucrose-P-Synthase Activity in Transformed Plants

Attempts to overexpress spinach SPS in potato have been largely thwarted by co-suppression phenomena. Similarly, replacement of serine residue 162, modifies the light/dark regulation of SPS but does not enhance total foliar SPS activity. In contrast, overexpression of a maize SPS cDNA in tomato or *Arabidopsis* leaves leads to substantial increases in total foliar SPS activity in both the light and dark. In the following studies SPS overexpression did not lead to a change in maximal extractable foliar NR activity or the C/N ratio in comparison to the controls when plants were grown in air or with CO$_2$ enrichment.

Overexpression of a maize SPS cDNA under the control of the promoter for the small sub-unit of Rubisco (rbcS; Worrell et al., 1991), increased foliar SPS activity between 300–600%. SPS activity was also increased in the roots of the rbcS transformants (Galtier et al., 1993). Increased foliar SPS activity was also observed when the maize leaf SPS cDNA was expressed under the control of the CaMV 35S promoter (Ferrario-Méry et al., 1997) or when the rbcS-SPS construct was expressed in *Arabidopsis* (Signora et al., 1998). Overexpression of an SPS from a C$_4$ plant, maize, hence resulted in substantial increases in foliar SPS activity and an increased capacity for sucrose synthesis in the transformed C$_4$ plants (Worrell et al., 1991; Galtier et al., 1993, 1995; Micallef et al., 1995; Signora et al., 1998). The marked increase in the maximum extractable foliar SPS activities in these transformants may be due to incomplete regulation of the introduced SPS protein by protein phosphorylation. Unlike the native tomato or *Arabidopsis* SPS forms, the introduced maize enzyme did not appear to be inactivated in darkness (Worrell et al., 1991; Galtier et al., 1993, 1995; Signora et al., 1998).

While Micallef et al. (1995) and Signora et al. (1998) found no statistically significant differences in photosynthesis between the untransformed controls and the transformants in air, Galtier et al. (1993, 1995) found that photosynthetic CO$_2$ assimilation was increased in leaves of the transformed tomato plants in air relative to controls. The light and CO$_2$-saturated rates of photosynthesis were increased by as much as 20% relative to the untransformed controls (Galtier et al., 1993, 1995). When plants are exposed to CO$_2$ enrichment, CO$_2$ assimilation can exceed end-product (triose phosphate) utilization (Stitt, 1986). The increased capacity for sucrose synthesis afforded by SPS overexpression can alleviate this feedback inhibition (Signora et al., 1998).

There was a strong positive correlation between foliar SPS activity and the ratio of sucrose to starch in the leaves when plants overexpressing SPS were grown in air or with CO$_2$ enrichment (Galtier et al., 1993, 1995; Signora et al., 1998). Improved rates of photosynthesis at elevated CO$_2$ and modification of foliar carbon partitioning in favor of sucrose, may therefore be regarded as common features of SPS overexpression (Galtier et al., 1993, 1995; Micallef et al., 1995; Signora et al., 1998). While SPS overexpression did not affect the yield of tomato fruit, it affected both the import and turnover of sucrose in the fruit of the transformed plants (Nguyen-Quoc et al., 1999).

### III. Phosphoenolpyruvate Carboxylase

PEPC plays an important role in C$_4$ and CAM plants as the primary carboxylating enzyme of photosynthesis and in C$_3$ plants functions broadly in pH regulation (the ‘pH stat’), stomatal opening and nitrogen assimilation. The enzyme is generally
regulated by feedback inhibition by malate and allosteric activation by Glc-6-P, and these effects are often most pronounced at suboptimal pH (Chollet et al., 1996). Phosphorylation activates PEPC (Chapter 18, Fig. 3, Furbank et al.) by modulating sensitivity to metabolic effectors, in particular, inhibition by malate. Given that the N-terminal phosphorylation domain is strictly conserved, it is quite likely that protein phosphorylation is an important mechanism regulating higher plant PEPC, including the C₃ leaf enzyme (Li et al., 1996).

A. Regulation of Phosphoenolpyruvate Carboxylase-Kinase Activity

It is thought that PEPC is phosphorylated by one or more highly specific protein kinases, that are Ca²⁺-independent and composed of 30- to 39-kDa polypeptides. The PEPC-kinase(s) are induced by light in C₃ leaves, and the induction is blocked by cycloheximide, as has been extensively studied in C₄ leaves and other systems. Studies with isolated C₄ mesophyll cells have identified a complex light-signal transduction pathway that involves increased cytosolic pH and [Ca²⁺]. In situ, the mechanism of C₃ PEPC-kinase upregulation appears to differ from that of C₄ systems by an apparent requirement for Gln.

B. Manipulation of Phosphoenolpyruvate Carboxylase Activity in Transformed Plants

The possibility of transferring characteristics of the C₃ isofoms to C₄ plants have been evaluated using hybridization techniques involving C₃ and C₄ plants and also by direct genetic manipulation. Expression of C₄ type PEPC cDNAs from maize in a C₃ plant, tobacco, have been achieved using either constitutive expression under the control of the 35S promoter (35S-PEP transformants; Kogami et al., 1994) or expression specific to photosynthetic tissues the N. plumbaginifolia cab (chlorophyll a/b binding protein) promoter (Hudspeth et al., 1992). Aberrant transcription initiation was observed when expression was under the control of the native promoter of the maize PEPC gene but expression of PEPC transcripts remained light-regulated (Hudspeth et al., 1992). When the cab gene promoter was used, expression of the transgene was observed only in the mesophyll cells. Total foliar PEPC activity in the transformants was twice that of the untransformed plants. In addition, two novel forms of PEPC with different kinetic properties (low and high Kₘ values for PEPC) were identified in the transformants (Hudspeth et al., 1992). The transformants had higher foliar malate contents than the untransformed controls but photosynthesis was comparable in all plants. An increase in total extractable foliar PEPC activity and two forms of PEPC were also observed in the 35S-PEPC transformants (Kogami et al., 1994) but in this case phenotypic effects were observed in the transformed plants (which had a lower growth rate and less chlorophyll).

When a bacterial PEPC cDNA was expressed in potato the growth rates of the transformed plants was decreased (Gehlen et al., 1996) without any changes in photosynthetic rate. In this case malate accumulated in the leaves but no other changes were reported. When the PEPC cDNA was expressed in the antisense orientation malate accumulation in the leaves was decreased but no changes in photosynthesis or growth rate were observed (Gehlen et al., 1996).

PEPC is encoded by a gene family, each member of which exhibits differential expression in leaf mesophyll (C₃ plants), bundle sheath (C₄ plants), roots or nodules. Several studies have therefore attempted to identify the main regions of the promoter determining tissue specificity and light inducibility. The expression of the complete coding sequence of a C₄ type PEPC gene from Sorghum vulgare flanked by the 5’ upstream sequence and the 3’ sequence, was obtained in transformed tobacco plants (Tagu et al., 1991). This study showed that a promoter from a monocotyledonous species was effective in a dicotyledonous plant and also allowed correct excision of introns in the gene. Moreover, transgene mRNA was not detected in roots of the transformants, suggesting a role for the 5’ region of the promoter in tissue-specific expression. Evidence that the 5’ untranslated region of the PEPC gene is of great importance in nodule-specific expression (Pathirana et al., 1997), in salt-stress inducibility in CAM plants (Cushman et al., 1993) and in the C₄-specific expression (Stockhaus et al., 1994) was provided by expression of a GUS reporter gene fused to different PEPC promoter regions in transformed tobacco, alfalfa and rice. Similar homology between light-regulated expression was observed in C₃ and C₄ plants (Matsuoka et al., 1994).

Site-directed mutagenesis of recombinant Sorghum PEPC was used to study the role of the highly conserved Ser residue which is involved in reversible
phosphorylation/dephosphorylation reactions controlling light/dark modulation of enzyme activity. The results obtained from such studies are in agreement with those obtained in other studies on structure/function and phylogenetic relationships. These data suggest that the differences between PEPC activities in plants arise from differential control of gene expression rather than variations in the primary structure of the isoforms.

IV. Nitrate Reductase

Control of NR by phosphorylation is a two-step process. The inactivation of NR by covalent modification involves: i) phosphorylation of Ser-543; and ii) binding of a 14-3-3 inhibitor protein to phospho-NR in the presence of a divalent cation. The regulatory phosphorylation site, Ser-543, is contained in the hinge 1 region that connects the heme and molybdenum-cofactor domains. Phosphorylation of Ser-543 alone has no effect on NR activity but rather completes the motif required for binding of the 14-3-3 protein (Huber et al., 1996; Moorhead et al., 1996). The amino acid sequence surrounding the phosphorylation site (R-T-A-phosphoSer) matches closely the consensus motif for 14-3-3 protein binding (R-S-X-phosphoSer-X-P) originally identified by Muslin et al., (1996). The 14-3-3 proteins are small acidic proteins that are ubiquitous among eukaryotes and are generally highly conserved. In plants they are present in small gene families, and by analogy to animal systems, are thought to function in regulation of a wide array of regulatory and signaling pathways. In addition to their role in NR regulation, they apparently also serve as the fusicoccin receptor and may also function in the regulation of the plasma membrane H+ -ATPase.

A. Nitrate Reductase Protein Kinases and Protein Phosphatase

Spinach leaves contain several protein kinases that can phosphorylate a synthetic peptide based on Ser-543 and can also phosphorylate this residue in the native protein. Two of the enzymes are Ca2+-dependent (Bachmann et al., 1996; Douglas et al., 1996) and thus, cytosolic [Ca2+] may be involved in regulating NR phosphorylation. In addition, other potential regulators of the NR-kinase(s) are metabolic intermediates such as DHAP, Glc-6-P, and Fru-1,6-P2 (Bachmann et al., 1996). Inhibition of NR-kinase activity by metabolites may be one of the components of the light-signal transduction pathway that mediates the light activation of NR. It is not known whether the metabolites interact with the kinase(s) and/or the target protein.

The endogenous protein phosphatase(s) that dephosphorylate and activate phospho-NR are thought to belong to the PP2A family, but little is known about the native enzymes that function in vivo. As discussed above, the 14-3-3 inhibitor protein that inactivates phospho-NR binds directly to the regulatory phosphorylation site (Ser-543). As a result, accessibility of the phospho-serine to phosphatases is reduced (Bachmann et al., 1996). This may have two important consequences. First, it may explain why the rate of NR activation in vivo is often slower than the rate of inactivation. Second, it may explain how chelation of Mg2+ or addition of Pi or salt, stimulate NR activation in vitro. It appears that Mg2+ binding to the 14-3-3 protein is required in order for the inhibitor protein to bind to phospho-NR (Athwal et al., 1998). The binding of the 14-3-3 protein is also affected by high ionic strength or Pi. Conditions that weaken binding would be expected to increase accessibility of phosphoSer-543 to phosphatases and thereby stimulate the rate of dephosphorylation.

B. Nitrate Reductase Hysteresis

Hysteresis refers to slow changes (e.g. in protein conformation) that affect the catalytic activity of an enzyme. Hysteric behavior of NR was first described with purified NR in vitro by Lillo and Ruoff (1992) in reference to a time-dependent activation by pyridine nucleotides. It was subsequently shown that NR extracted from leaves placed in the dark for several hours had lower activity than when extracted from illuminated leaves, even when assays were performed in the absence of Mg2+ to eliminate inactivation by phosphorylation. The surprising observation was that NR activity in the dark extracts could be increased to the light activity by preincubation of extracts at 25 °C with one or more ‘activators,’ including Pi, AMP and pyridine nucleotides (Huber and Huber, 1995). The increase in activity was not blocked by protein phosphatase inhibitors indicating that dephosphorylation of Ser-543 was not involved. Rather, the activation may reflect the hysteretic behavior of the enzyme. Recent results suggest that it is only the phosphorylated form of NR that can adopt
the conformation with reduced activity, as assayed in the absence of Mg$^{2+}$ (Kaiser and Huber, 1997). Although not demonstrated experimentally, the postulated less active conformation of NR may involve a tightened binding of the 14-3-3 inhibitor protein that is semi-stable in the absence of Mg$^{2+}$.

Conversion to the active conformation (hysteresis) in vitro is stimulated by a variety of 'activator compounds.' The process also seems to occur in vivo and may also involve effectors. In addition to activators, amino acids may serve as negative effectors that can block the activation in situ. Thus, feeding amino acids to excised leaves via the transpiration stream largely prevented the increase in NR activity that can be observed when assays are performed in the absence of Mg$^{2+}$. Thus, amino acids may effectively reduce NR activity in vivo by affecting the hysteretic behavior of the enzyme. It should also be noted that high concentrations of amino acids are also known to reduce NR gene expression (see Section I), and would likely restrict de novo NR protein synthesis.

C. Manipulation of Nitrate Reductase Activity in Transformed Plants

When the NR-deficient mutant of *N. plumbaginifolia* was transformed with a full-length tobacco NR cDNA fused to the CaMV 35S promoter, the transformed plants were phenotypically comparable to the untransformed controls (Vincentz and Caboche, 1991; Foyer et al., 1993, 1994). Constitutive NR expression caused a two-fold increase in maximal extractable NR activity, a decrease in the foliar NO$_3^-$ content and an increase in the total amino acid (largely due to an increase in the glutamine pool) contents without any changes in total N, soluble sugars or starch (Foyer et al., 1994; Quilleré et al., 1994). The observed increase in the glutamine to glutamate ratio and the glutamine to sucrose ratio in the 35S-NR plants had no apparent effect on enzymes (SPS, PEPC, GS) were not modified (Ferrario-Méry et al., 1997). In hydroponically-grown plants NR activity, NO$_3^-$ and amino acid levels were not decreased following CO$_2$ enrichment. NR mRNA was decreased, however, and glutamine was increased in all plants grown at high CO$_2$ even though NR activity and NO$_3^-$ levels were similar to those of plants grown in air. The NR activation state did not vary in response to CO$_2$ enrichment suggesting that the phosphorylation/dephosphorylation state of the protein was not responsive to CO$_2$ enrichment.
The terminal region is not particularly well conserved and acidic residues in the N-terminal regions could provide sites of electrostatic interaction with other proteins. An internal deletion of 56 amino acids in the N-terminal MoCo domain of the NR protein (ΔNR) was performed to determine whether this region is involved in the post-translational regulation of the NR protein, for example, in proteolysis, inactivation by protein phosphorylation, or association with the NR protein inhibitor. The resultant transformants contained substantial NR activity and displayed normal growth characteristics comparable to the untransformed controls but, similar to the 35S-NR transformants, the ΔNR gene was not regulated at the transcriptional level. In addition, foliar NR activity was no longer regulated by light at the post-transcriptional level, the activation state of the enzyme remaining constant during light/dark transitions. The NR protein was always in the activated form (Nussaume et al., 1995). Interestingly, the ΔNR deletion does not include the serine residue involved in protein phosphorylation. This site occurs in the hinge region of the NR protein which separates the molybdenum cofactor and the heme domains in spinach (Ser-543) and in Arabidopsis (Ser-534; Bachmann et al., 1996; Su et al., 1996). Whether phosphorylation of the protein still occurs in the ΔNR protein or whether linkage to the NR protein inhibitor is impaired by the N-terminal deletion remains unresolved. Diurnal fluctuations in total foliar amino acid pools, particularly Gln, which are observed in the untransformed plants, are damped in the ΔNR plants. Experiments using $^{15}$N incorporation to determine nitrogen assimilation rates have not yet been performed. It is unclear whether constitutively active NR will lead to increased rates of N assimilation; assimilation at night may be limited, for instance, by reducing power availability in the absence of photosynthetic electron transport.

Such studies demonstrate the complexity of NR regulation at the transcriptional, post-transcriptional and post-translational levels. These regulatory mechanisms act in parallel to determine the overall leaf rate of $\text{NO}_3^-$ reduction. The metabolic factors acting at each of these steps have not been clearly identified. $\text{NO}_3^-$, N metabolites such as glutamine, and carbohydrates such as sucrose, are involved in induction of NR gene transcription and may also be involved in turnover of the NR mRNA and NR protein. The relative importance of each of these regulatory devices may vary from species to species; NR is inactivated in the absence of $\text{NO}_3^-$ in transformed Chlamydomonas constitutively expressing NR.
Plants modified in GS and GOGAT activities have proved to be valuable tools for studying the control of photosynthetic and photorespiratory C/N interactions (Häusler et al., 1994a,b; Leegood et al., 1995, 1996) as well as to study the effects of metabolic intermediates on gene expression (Dzuibany et al., 1998). Screening procedures designed to isolate photorespiratory mutants in *Ambidopsis*, barley and pea (Somerville, 1986; Blackwell et al., 1988a,b) also identified plants modified in GS and GOGAT activities. These mutants were recognized by their ability to grow with a comparable phenotype to the wild-type under non-photorespiratory conditions of atmospheric CO$_2$ enrichment but showed severe stress symptoms in air where photorespiration occurs (Blackwell et al., 1988b). These mutants are unable to reassimilate photorespiratory NH$_4^+$ when exposed to air photosynthetic CO$_2$ assimilation is inhibited, foliar NH$_4^+$ increases and Gln rapidly accumulates at the expense of almost all other amino acids. Since metabolism in these mutants has been extensively reviewed elsewhere (Leegood et al., 1995, 1996) the following sections describe results obtained from the analysis of transformed plants only.

Transformed tobacco plants expressing a cytosolic GS cDNA from alfalfa, under the control of the 3SS promoter, were obtained by Eckes et al. (1989) and Temple et al. (1993). The abundance of the mRNA derived from the transgene was 10 times higher than that measured in untransformed alfalfa plants. Maximal extractable foliar GS activity were enhanced in the transformants by 10–25% (Temple et al., 1993) or by 500% (Eckes et al., 1989). The NH$_4^+$ content of the leaves of the transformants was drastically decreased but the amino acid composition was unchanged (Eckes et al., 1989). In one case a 45% increase in the total soluble protein content of the leaves was observed (Temple et al., 1993). Growth and seed production of the transformed plants were comparable to those of the controls. The transformants were 20-fold more resistant in vitro to the GS inhibitor, phosphinothricin (Eckes et al., 1989).

Overexpression of cytosolic GS in transformed tobacco constitutively expressing cytosolic or chloroplastic GS cDNAs correlated with increased growth whereas in cases where co-suppression was observed growth was inhibited (Lam et al., 1995). Transformation of the legume *Lotus corniculatus* L. to overexpress a soybean cytosolic GS cDNA (Vincent et al., 1997) led to increases in total foliar GS activity of up to 80%. When these transformants were grown on 12 mM NH$_4^+$ greater increases in foliar amino acids and ammonium were observed in leaves and roots while decreases in carbohydrates in roots were more pronounced in transformants than in the untransformed controls. Increases in amino acid contents appeared to arise because of modified shoot protein degradation and to an early senescence and floral development in transformed *L. corniculatus*.

A role of cytosolic GS in the vascular tissue in nitrogen assimilation and transport was suggested in transformed alfalfa overexpressing a cytosolic GS gene in the antisense orientation under the control of a phloem specific promoter (Temple et al., 1993). The only transformants which remained viable were those where minimum suppression of GS was achieved. Expression of two alfalfa cytosolic GS cDNAs differing in their 3’ untranslated regions and fused to either vascular or nodule-specific promoters from *Arabidopsis thaliana* or soybean in alfalfa resulted in down-regulation of GS in the nodule isoform but increased in the stem (Temple and Sengupta-Gopalan, 1997).

Transformed tobacco plants with decreased GS expression in the phloem and roots contained less proline indicating that GS in the phloem plays a major role in the regulation of proline synthesis. Despite the low nucleotide homology between alfalfa GS1 and tobacco GS2 genes the decrease in GS activity in the transformants was caused by inhibition of the chloroplastic GS2 only. Decreased GS activity was accompanied by a 40% decrease in total soluble protein and decreases in PEPC and hydroxypyruvate

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**Chapter 8 Regulation of Carbon Fluxes in the Cytosol**

(V. Glutamine Synthetase and Glutamate Synthase)

Plants modified in GS and GOGAT activities have proved to be valuable tools for studying the control of photosynthetic and photorespiratory C/N interactions (Häusler et al., 1994a,b; Leegood et al., 1995, 1996) as well as to study the effects of metabolic intermediates on gene expression (Dzuibany et al., 1998). Screening procedures designed to isolate photorespiratory mutants in *Ambidopsis*, barley and pea (Somerville, 1986; Blackwell et al., 1988a,b) also identified plants modified in GS and GOGAT activities. These mutants were recognized by their ability to grow with a comparable phenotype to the wild-type under non-photorespiratory conditions of atmospheric CO$_2$ enrichment but showed severe stress symptoms in air where photorespiration occurs (Blackwell et al., 1988b). These mutants are unable to reassimilate photorespiratory NH$_4^+$ when exposed to air photosynthetic CO$_2$ assimilation is inhibited, foliar NH$_4^+$ increases and Gln rapidly accumulates at the expense of almost all other amino acids. Since metabolism in these mutants has been extensively reviewed elsewhere (Leegood et al., 1995, 1996) the following sections describe results obtained from the analysis of transformed plants only.

Transformed tobacco plants expressing a cytosolic GS cDNA from alfalfa, under the control of the 3SS promoter, were obtained by Eckes et al. (1989) and Temple et al. (1993). The abundance of the mRNA derived from the transgene was 10 times higher than that measured in untransformed alfalfa plants. Maximal extractable foliar GS activity were enhanced in the transformants by 10–25% (Temple et al., 1993) or by 500% (Eckes et al., 1989). The NH$_4^+$ content of the leaves of the transformants was drastically decreased but the amino acid composition was unchanged (Eckes et al., 1989). In one case a 45% increase in the total soluble protein content of the leaves was observed (Temple et al., 1993). Growth and seed production of the transformed plants were comparable to those of the controls. The transformants were 20-fold more resistant in vitro to the GS inhibitor, phosphinothricin (Eckes et al., 1989).

Overexpression of cytosolic GS in transformed tobacco constitutively expressing cytosolic or chloroplastic GS cDNAs correlated with increased growth whereas in cases where co-suppression was observed growth was inhibited (Lam et al., 1995). Transformation of the legume *Lotus corniculatus* L. to overexpress a soybean cytosolic GS cDNA (Vincent et al., 1997) led to increases in total foliar GS activity of up to 80%. When these transformants were grown on 12 mM NH$_4^+$ greater increases in foliar amino acids and ammonium were observed in leaves and roots while decreases in carbohydrates in roots were more pronounced in transformants than in the untransformed controls. Increases in amino acid contents appeared to arise because of modified shoot protein degradation and to an early senescence and floral development in transformed *L. corniculatus*.

A role of cytosolic GS in the vascular tissue in nitrogen assimilation and transport was suggested in transformed alfalfa overexpressing a cytosolic GS gene in the antisense orientation under the control of a phloem specific promoter (Temple et al., 1993). The only transformants which remained viable were those where minimum suppression of GS was achieved. Expression of two alfalfa cytosolic GS cDNAs differing in their 3’ untranslated regions and fused to either vascular or nodule-specific promoters from *Arabidopsis thaliana* or soybean in alfalfa resulted in down-regulation of GS in the nodule isoform but increased in the stem (Temple and Sengupta-Gopalan, 1997).

Transformed tobacco plants with decreased GS expression in the phloem and roots contained less proline indicating that GS in the phloem plays a major role in the regulation of proline synthesis. Despite the low nucleotide homology between alfalfa GS1 and tobacco GS2 genes the decrease in GS activity in the transformants was caused by inhibition of the chloroplastic GS2 only. Decreased GS activity was accompanied by a 40% decrease in total soluble protein and decreases in PEPC and hydroxypyruvate
reductase activities. Plants with low GS were comparable to untransformed controls and did not suffer from \( \text{NH}_4^+ \) accumulation when plants were grown under photorespiratory conditions. Expression of the antisense construct in alfalfa led to a decrease in native alfalfa GS (Temple and Sengupta-Gopalan, 1997).

Tobacco transformants with Fd-GOGAT activities of between 36% and 83% were grown with CO\(_2\) enrichment (4000 ppm) to prevent photorespiration and to avoid toxic \( \text{NH}_4^+ \) accumulation. In these conditions, the growth rate was similar to that measured in the untransformed controls (Fig. 4). Following transfer to air, the transformants produced chlorotic symptoms on the leaves and development was reduced (Hirel et al., 1997). This suggests that a 60% decrease in Fd-GOGAT activity did not limit primary nitrogen assimilation and confirmed the role of Fd-GOGAT in the recycling of photorespiratory \( \text{NH}_4^+ \) in agreement with results obtained in studies of photorespiratory mutants deficient in GS (Blackwell et al., 1987; Wallsgrove et al., 1987) or Fd-GOGAT activities (Somerville and Ogren, 1980; Kendall et al., 1986; Lea et al., 1992). These mutants were only viable when grown in \( \text{CO}_2\)-enriched atmospheres. Following transfer to air, these mutants showed large increases in the Gln and \( \text{NH}_4^+ \) contents of the leaves together with decreases in other amino acids and an inhibition of \( \text{CO}_2\) assimilation.

Transformed plants with intermediate levels of GOGAT activity allow the study of long-term effects on nitrogen and carbon assimilation, particularly the impact of changes in metabolite contents on the regulation and expression of key enzymes. After four days exposure to photorespiratory conditions, increased foliar \( \text{NH}_4^+ \) accumulation was observed in the leaves of the transformants with the lowest Fd-GOGAT activity. This was accompanied by large increases in other metabolites, especially Gln and 2-OG. In contrast Glu contents were lower in the transformants than in the untransformed controls. No change in foliar Glu content was observed following the transfer to air, suggesting that an alternative pathway of Glu production was activated in the transformants. Such an alternative may be provided by GDH, whose activity was increased in the transformants. Although this enzyme has a relatively low affinity for \( \text{NH}_4^+ \) and 2-OG, these properties could be less restricting in the transformants, where levels of these compounds are increased. It is possible that these high levels of 2-OG and \( \text{NH}_4^+ \) are, at least partly, responsible for GDH induction. A correlation between low Fd-GOGAT activity and high NR and NiR transcript abundance was observed in transformed leaves but neither foliar NR nor NiR activities were changed. The increases in NR and NiR transcript abundance are surprising since both Gln and \( \text{NH}_4^+ \) accumulate in the leaves of the transformants. Interestingly, significant correlations were established between the ratio of Gln to 2-OG and the abundance of NR and NiR transcripts, suggesting that 2-OG could prevent the inhibitory effect of glutamine on NR and NiR transcription. The abundance of transcripts for a high affinity \( \text{NO}_3^- \) transporter and for NR were decreased in the roots of the Fd-GOGAT transformants, even though the only metabolite modification observed in roots was a large increase in \( \text{NH}_4^+ \). \( \text{NH}_4^+ \) or low Fd-GOGAT may thus influence shoot to root interactions. High shoot Gln and \( \text{NH}_4^+ \) contents may convey information suggesting greater abundance of reduced N in leaves. This would lead to a decrease in N uptake and in primary N assimilation in the roots. Gln also induces increased PEPC activity but PEPC transcript abundance was unchanged. Gln accumulation alone may not elicit an appropriate response and while parallel increases in Gln and 2-OG are probably uncommon in plants, they may be required to elicit appropriate responses in extreme stress situations (Fig. 5).
VI. Plasma Membrane H⁺-ATPase

The plasma membrane H⁺-pumping ATPase functions to generate a pH and electrical gradient that drives ion uptake, and is involved in a variety of processes including nutrient uptake, osmoregulation and pH control. A major recent development is that the activity of the pump may also be controlled by phosphorylation in a mechanism that also involves 14-3-3 proteins.

A. Regulation by Phosphorylation

Recent evidence suggests that the pump is inactivated by phosphorylation and binding of a 14-3-3 protein (Moorhead et al., 1996), which also serves as the fusicoccin (FC) receptor (for review see De Boer, 1997). Activation of the ATPase can be achieved by dephosphorylation catalyzed by PP2A or dissociation of the ATPase: 14-3-3 complex with phoshoSer-259-Raf-1 peptide (Moorhead et al., 1996), which conforms to the 14-3-3 binding motif (Muslin et al., 1996). The pump can also be activated with FC, and although different models exist for the mechanism of activation, both involve displacement of the 14-3-3 proteins from at least one of its binding sites on the ATPase. Concerted regulation of NR and the H⁺-ATPase (which provides the driving force for nitrate uptake) by a common mechanism involving 14-3-3s, suggests the potential for coordinate regulation but details remain to be elucidated.

VII. Conclusions

C and N assimilation are closely coordinated in leaves; if CO₂ is removed from the air surrounding an illuminated leaf N assimilation ceases soon after photosynthesis is inhibited. Similarly, a strong correlation between maximal extractable NR activity...
and ambient photosynthesis was found in tobacco and maize in well-watered and droughted plants (Ferrario-Méry et al., 1998). The coordination of the pathways of carbon and nitrogen assimilation in plants involves the concerted action of a repertoire of signals that allow graded molecular and physiological responses. A large number of studies have concerned the mechanisms controlling the expression of genes encoding the enzymes of N assimilation (NR, NiR, GS and GOGAT) and the regulation of NR activity by protein phosphorylation. In contrast, relatively few signal molecules coordinating the C/N interaction have been identified and fundamental questions regarding mechanism remain unanswered. Sugars (such as sucrose and glucose) and 2-OG are involved in the transcriptional and post-transcriptional regulation of enzymes associated with N assimilation, while nitrate and amino acids modulate the expression and activation state of enzymes of C metabolism. Such metabolites convey information on carbohydrate and nitrogen status throughout the plant to allow rapid and appropriate adaptive responses to environmental and metabolic stimuli. Protein phosphorylation regulates key enzymes of N assimilation, sucrose biosynthesis and the synthesis of organic and amino acids, ensuring coordination of the pathways of C and N assimilation and the anaplerotic pathway which provides carbon skeletons for amino acid biosynthesis (Fig. 3). It is possible that pathways of signal transduction for the regulation of GS similar to that observed in E. coli, occur in plants. This involves 4 proteins including PII, which control the transcription of the GS gene (Atkinson et al., 1994).

Nitrate, per se, has been shown to be a metabolic signal that enhances transcription of several genes including those encoding PEPC, cytosolic pyruvate kinase, mitochondrial citrate synthase, and cytosolic isocitrate dehydrogenase (Scheible et al., 1997c). Adequate N-nutrition increases enzymes necessary for nitrate reduction and amino acid biosynthesis and favors enhanced rates of photosynthesis. Nitrate also directly represses expression of the large subunit of ADP-glucose pyrophosphorylase, and this will tend to reduce the partitioning of carbon into starch in N-replete leaves (Fig. 1). N assimilation also increases PEPC activity and decreases SPS activity favoring decreased rates of sucrose synthesis and enhanced C flow through the anaplerotic pathway (Fig. 3).

At least two important C/N interactions, regulated by protein phosphorylation, in the N-sufficient leaves involve light-activation of enzymes. Firstly, Gln is required for the light activation of PEPC-kinase in C4 leaves (Champigny and Foyer, 1992). This enzyme phosphorylates and activates PEPC (Li et al., 1996). Thus, PEPC will only be activated when N-nutrition is adequate. Second, light activation of NR (by dephosphorylation of Ser-543) requires photosynthesis and metabolites that inhibit NR-kinase, such as DHAP, are probably involved. Thus, nitrate will only be reduced when C-metabolites are available for incorporation into amino acids.

N-deficiency causes inhibition of photosynthesis and hence the synthesis of carbohydrates. However, low nitrate, per se, is a signal for increased expression of ADPGlc PPase and thereby increases the capacity for starch synthesis. In addition, the accumulation of PGA, that is characteristic of N-deficiency, will increase ADP-glucose production by allosteric activation of ADPGlc PPase (Scheible et al., 1997c). The low levels of amino acids that are found in N-deficient leaves may also have other metabolic effects. Firstly, the light activation PEPC-kinase will be inhibited and hence organic acid synthesis will be reduced. Secondly, foliar amino acids drop to values low enough to restrict protein synthesis, inhibition of SPS activation may also be observed. Thirdly, since N-deficiency reduces photosynthesis, decreased metabolite availability (especially Glc 6-P) and elevated Pi will also contribute to a reduction in SPS activation. Thus, N-deficient leaves will tend to partition more C into starch and less into organic acids, amino acids and sucrose.

A decrease in assimilate export will cause accumulation of assimilates such as sucrose and amino acids in leaves. A build-up of amino acids has been postulated to inhibit SPS-PP(s) and thereby reduce SPS activation, resulting in increased diversion of C into starch. The increases in metabolites in the cytosol observed when SPS is inactivated in the light will inhibit NR-kinase(s) and result in ‘hyper-activation’ of NR. Thus, nitrate reduction will continue or perhaps even increase in the short-term. In the long-term, nitrate reduction and amino acid biosynthesis cannot continue unabated for at least two reasons. Firstly, amino acid accumulation in leaves will restrict NR gene expression (Vincentz et al., 1993), and thus restrict de novo synthesis of NR enzyme protein. Secondly, amino acid accumulation may prevent the hysteretic activation of NR that is postulated to involve conformational changes (Huber
et al., 1996). Thus, nitr ate assimilation will be restricted even in the presence of sufficient nitr ate.

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Starch Metabolism in Leaves

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Summary

Starch is a carbohydrate reserve used by many higher plants. Transitory starch is synthesized in the chloroplasts of higher plants both as an overflow for newly-assimilated carbon and as a reserve of carbohydrate for periods of darkness when photosynthesis is not possible. Starch is present in the chloroplast as insoluble granules consisting of two types of glucose polymer: amylopectin and amylose. The pathway of synthesis has been well defined and involves the action of phosphoglucomutase, ADPglucose pyrophosphorylase, starch synthases, and branching enzymes. However, there are multiple isoforms of starch synthases and branching enzymes and the extent to which different isoforms play distinct roles in determining starch structure is not clear. The simultaneous action of degradative enzymes may also play a role in determining the structure of starch during its synthesis. It is likely that starch synthesis is regulated through the allosteric control of ADPglucose pyrophosphorylase. Many enzymes have been implicated in the process of transitory starch degradation although few have been confirmed to play a role in vivo. It is not clear whether the initial attack on the starch granules is hydrolytic—catalyzed by amylases—or phosphorolytic—catalyzed by starch phosphorylase. The route by which sugar is released from shorter soluble glucan chains also remains unclear. There is physiological evidence that the process of starch degradation is subject to regulation. However, given the uncertainty about the mechanism of starch degradation, it is not possible to develop hypotheses about its regulation. The products of degradation may be exported from the chloroplast as sugars or as triose phosphate, but there is evidence that sucrose synthesis in the cytosol at night is supported by the export from the chloroplast of sugars rather than triose phosphate.

I. Introduction

Starch is the most abundant carbohydrate reserve in plants: it has been estimated that in excess of 10⁸ tonnes are produced annually (Galliard, 1987). Two types of starch can be distinguished according to function: reserve starch and transitory starch. Reserve starch accumulates in organs of perennation and dispersal, and is localised in non-green plastids, called amyloplasts (Martin and Smith, 1995). Transitory starch is formed during photosynthesis in the chloroplasts of many higher plants, and serves as an important store of carbohydrate for periods of darkness when photosynthesis is not possible. In this chapter we will discuss the occurrence, importance, structure, synthesis, and degradation of transitory starch. We will pay particular attention to the physiological significance of transitory starch and discuss current ideas on how starch metabolism is regulated at the biochemical level.

II. The Occurrence and Function of Transitory Starch

The pattern of starch accumulation in leaves is often portrayed as a strong diurnal rhythm, in which starch is synthesized in relatively large amounts during the day and almost entirely degraded during the night. This is certainly the case in some well-studied experimental systems. Leaves of pea grown under an 18-h photoperiod, Arabidopsis grown under a 12-h photoperiod and spinach grown under a 9-h photoperiod all degraded 85% or more of the accumulated starch in the subsequent dark period (Stitt et al., 1978; Gerhardt et al., 1987; Lin et al., 1988b). However, the extent of starch accumulation and the magnitude of the diurnal change vary considerably. First, different species allocate very different proportions of their newly-assimilated carbon to starch. Many species do not accumulate starch in their chloroplasts under normal growth conditions. Sucrose or—in the Gramineae—fructans are stored in the vacuoles of mesophyll cells in these species. In other species, starch synthesis can account for anything from a few percent to a very substantial proportion of the newly-assimilated carbon. For example, leaves of annual meadow grass (Poa annua L.) partitioned most of their assimilated carbon into sucrose and fructans, and less than 2% into starch (Borland and Farrar, 1988) whereas in leaves of sugar beet (Beta vulgaris L.) 40% of assimilated carbon was partitioned into starch (Li et al., 1992b). Second, the extent of the diurnal change in starch content changes as the leaf ages in some species but not in others. The starch content of tobacco, soybean and cotton leaves increases as leaves age, and the

Abbreviations: 3-PGA – 3-phosphoglycerate; AGPase – ADPglucose pyrophosphorylase; GBSSI – granule-bound starch synthase I; PGM – phosphoglucomutase; P_{i} – inorganic phosphate; SS I – starch synthase I; SS II – starch synthase II; SS III – starch synthase III; TPT – triose-phosphate translocator
magnitude of the diurnal change decreases (Matheson and Wheatley, 1962, 1963; Chang, 1979; Franceschi and Giaquinta, 1983), but there is little change in the diurnal pattern of starch accumulation and degradation through development in *Arabidopsis* leaves (Trethewey and ap Rees, 1994b). Third, the extent of starch accumulation may vary from one cell type to another within a single leaf. The parenchymatous bundle sheath of barley leaves contains four distinct types of photosynthetic cell, each of which displays different patterns of starch accumulation and degradation (Williams et al., 1986; Lin et al., 1988b). The relative amounts of starch in the two palisade layers and the spongy mesophyll layer of soybean leaves changed considerably during the photoperiod (Franceschi and Giaquinta, 1983). Fourth, starch content and the extent of the diurnal change are highly dependent upon the conditions in which the plant is grown—for example, the length of the photoperiod, the photon flux density and the night temperature (Hewitt et al., 1985; Fondy et al., 1989). Deficiencies of nitrogen and potassium and elevated levels of carbon dioxide usually result in increased accumulation of starch in leaves (Rufty et al., 1988; Farrar and Williams, 1991; Paul and Stitt, 1993). Attack by fungal and viral pathogens may also lead to accumulation of starch. This accumulation may be specifically in or around infected cells (Scholes and Farrar, 1987; Técsi et al., 1994), or generally throughout the leaves of an infected plant (Técsi et al., 1992).

The importance of transitory starch in those plants in which it accumulates is demonstrated by the phenotypes of mutant and transgenic plants in which accumulation is prevented or its magnitude is altered. Mutations in *Arabidopsis* and tobacco that eliminate activity of plastidial phosphoglucomutase (PGM; tobacco NS458, NS514, Hanson and McHale (1988); *Arabidopsis* TC-7, Caspar et al. (1986)) or ADPglucose pyrophosphorylase (AGPase; *adg2* mutant of *Arabidopsis*, Lin et al., 1988b) prevent accumulation of starch in the leaves. Growth of these mutants is indistinguishable from that of wild-type plants under long photoperiods. In the tobacco mutants, sucrose is synthesized and exported during the light period at a higher rate than in wild-type plants, compensating for the lack of sucrose synthesis from starch at night (Huber and Hanson, 1992; Geiger et al., 1995). However, when grown with long dark periods this compensatory effect is not adequate to support growth rates comparable with those of wild-type plants: all of the mutants exhibit reduced growth rates (Caspar et al., 1986; Lin et al., 1988b; Hanson and McHale, 1988; Schulze et al., 1991; Huber and Hanson, 1992). Indeed, a mutant of *Arabidopsis* lacking plastidial phosphoglucomutase also exhibited a late-flowering phenotype in days shorter than 16 hours (Corbesier et al., 1998). Transgenic plants in which the extent of accumulation of transitory starch has been altered also show compensatory changes in the diurnal pattern of sucrose export from the leaf. Potato plants in which the activity of the triose-phosphate translocator (TPT) in the chloroplast envelope has been reduced through the expression of antisense RNA have increased rates of transitory starch synthesis and reduced rates of sucrose synthesis and export during the day. This is compensated for by high rates of starch degradation leading to increased sucrose synthesis and export at night (Heineke et al., 1994). Potato plants in which the synthesis of transitory starch is decreased through expression of an antisense RNA for AGPase display increased rates of sucrose synthesis and export during the day, compensating for the reduction in sucrose synthesis from starch at night (Leidreiter et al., 1995).

Consideration of conditions which promote the accumulation of starch in leaves has given rise to two opinions about its function. First, transitory starch synthesis may occur primarily as an overflow for newly-assimilated carbon when assimilation exceeds the demand for sucrose (Stitt and Quick, 1989). The sequestration of carbon as insoluble starch allows high rates of assimilation to be maintained when the capacity to store and to export sucrose has been reached. Recent studies on transgenic plants with reduced rates of transitory starch synthesis have demonstrated a positive correlation between the capacity for starch synthesis and the rate of photosynthesis at elevated carbon dioxide concentrations (Ludewig et al., 1998). In intact leaves of some species [for example pea (Stitt et al., 1978), sugar beet (Fondy and Geiger, 1982) and maize (Kalt-Torres and Huber, 1987)] the rate of starch synthesis is low in relation to that of sucrose during the early part of the day and rises later, in parallel with a rise in the sucrose content of the leaf. Further experimental support for the idea of transitory starch as an overflow is provided by the general observation that restriction of the export of carbon from the chloroplast or from the leaf leads to increased rates of starch synthesis. For example, starch synthesis is increased in transgenic potato plants in which activity of the TPT or a sucrose-proton translocator involved in phloem loading has been reduced (Heineke et al., 1994, Kühn et al., 1996), and in plants in which...
phloem loading has been impaired by expression of high activities of invertase in the apoplast (von Schaewen et al., 1990). Starch synthesis is also increased in leaves of Clarkia mutants with decreased activity of cytosolic phosphoglucosomerase and hence a reduced rate of sucrose synthesis (Neuhaus et al., 1989). In spinach leaf discs the rate of starch relative to sucrose synthesis increased as the sucrose content of the discs increased (Stitt et al., 1984).

Second, transitory starch may provide a source of carbon for growth during the following night. This view is supported by the compensatory changes in the pattern of sucrose synthesis and export that occur in mutants unable to synthesize transitory starch, and by the fact that growth of these plants is restricted under short-day regimes (see above). The importance of transitory starch to the growth of the plant is clearly dependent upon the general growth conditions: an inability to synthesize transitory starch is likely to restrict growth to a greater extent in conditions when carbohydrate supply is limiting growth (for example in high nitrogen) than in conditions where some other factor is more important in limiting growth (Schulze et al., 1991). The pattern of starch synthesis in several species is also not consistent with a role simply as an overflow mechanism. For example, starch synthesis can occur concurrently with sucrose synthesis at low rates of photosynthesis. In Clarkia xantiana Gray (Onagraceae) rates of starch and sucrose synthesis were approximately equal under both saturating and limiting light, even though the rate of photosynthesis was three times greater in saturating than in limiting light (Krucekeberg et al., 1989). The ratio of sucrose to starch synthesis in Phaseolus was constant over a wide range of light intensities and carbon dioxide concentrations except where the rate of photosynthesis was very low, when sucrose synthesis was preferentially maintained (Sharkey et al., 1985). In a number of species (soybean, spinach, maize, pangola (Digitaria decumbens Stent.), sugar beet (Chatterton and Silvius, 1981; Jablonski and Geiger, 1987), and potato (Lorenzen and Ewing, 1992)) the proportion of newly-assimilated carbon that is partitioned to starch varies inversely with the length of the photoperiod. These observations, and extensive studies of the allocation of newly-assimilated carbon to starch in leaves of sugar beet, have led Geiger and colleagues to suggest that partitioning to starch may be regulated by an endogenous circadian rhythm which ensures that the amount of starch synthesized during the day is commensurate with the demand for carbon during the following night (Li et al., 1992b).

Overall, it seems likely that transitory starch is synthesized both as an overflow for newly-assimilated carbon and as a source of carbon during the night. The relative importance of these two roles depends on the species and the growth conditions. The ways in which the partitioning of newly-assimilated carbon into starch may be regulated are discussed in Section IV.B.

III. The Structure of Transitory Starch

Very little is known about the composition and structure of the starch granules in chloroplasts: almost all research on starch structure has been on the starches of non-photosynthetic storage organs. We will summarise the present understanding of storage starches and discuss whether this picture is likely to be applicable to transitory starch.

Storage starch granules vary enormously between species in size and shape but are similar in important features of composition and structure. They consist of two distinct types of glucose polymer. About 20–30% of their mass is made up of amylose, a predominantly linear molecule consisting of between 500 and 10,000 α(1-4) linked glucose residues. The remaining 70–80% of the granule is amylopectin, a polymer consisting of relatively short chains of α(1-4) linked glucose residues, joined by α(1-6) linkages.

Microscopical studies and X-ray diffraction and scatter analyses indicate that granules of storage starches are semi-crystalline, containing concentric rings of alternating crystalline and amorphous lamellae with a repeat distance of 9 nm (French, 1984; Imberty et al., 1991; Jenkins et al., 1993: Fig. 1). This level of organization is a function of amylopectin rather than amylose molecules since it occurs in the storage starches of both wild-type plants and mutant plants unable to synthesize amylose. The existence of a 9-nm repeat has been explained by the fact that the distribution of chain lengths within the amylopectin molecule is polymodal, with maxima at chain lengths of approximately 12–16, about 40 and about 70 glucose residues (French, 1984; Hizukuri, 1986). It is believed that the amylopectin chains of 12–16 glucose residues are organised into clusters. Chains of 40 glucose residues span two clusters, and chains of 70 glucose residues span three clusters. Within clusters, adjacent chains form double
helices which pack together in ordered arrays, giving rise to the crystalline lamellae (Fig. 1). The amorphous lamellae are the zones in which a large number of α(1,6) linkages occur, hence few double helices are formed (Hizukuri, 1986; Jenkins et al., 1993). The crystalline and amorphous lamellae form concentric zones, usually several hundreds of nm wide, within the starch granule. These semi-crystalline zones alternate with amorphous zones in which the arrangement of the amylopectin molecules is not understood (Fig. 1).

The transitory starch granules of chloroplasts are generally discoid in shape and less than five microns across (Badenhuizen, 1969). Where it has been studied, considerable variation has been found in the size of transitory granules within a single leaf. For example, most of the granules in a sunflower leaf were 0.2 to 2.5 microns across, but about 3% of the granules were 4 to 5.5 microns across (Radwan and Stocking, 1957). In Pellionia leaves granules in the spongy mesophyll were much larger than those in the palisade layer (Weier, 1936). There is general agreement that transitory starch granules contain both branched polymers of high molecular mass and relatively unbranched polymers of lower molecular mass, corresponding approximately to the amylpectin and amylose components of storage starch. The ratio of amylose to amylpectin is lower in transitory than in storage starch, and the size and chemical structure of both of these components probably differs between transitory and storage starch. Around 16% or less of the starch of leaves of tobacco, sunflower, rice, pea and potato is amylose, whereas this value is almost invariably 20% or more for storage starches (Radwan and Stocking, 1957; Hovenkamp-Hermelink et al., 1988; Taira et al., 1991; Matheson, 1996; Tomlinson et al., 1997). The amylose content of the transitory starches of tobacco and cotton leaves increases as the starch content of the leaves increases through development (Chang, 1979; Matheson, 1996). The amylose of transitory starch is reported to be of lower molecular mass and more branched than that of storage starches (Radwan and Stocking, 1957; Matheson, 1996). The amylpectin of transitory starch has a polymodal distribution of chain lengths which approximates to that of storage starches (Matheson, 1996). However, a detailed study of the distribution of lengths of the shorter chains of amylpectins from the transitory and storage starches of pea revealed considerable differences between the two (Tomlinson et al., 1997). The amylpectin of the transitory starch had a very strong polymodal distribution of chain lengths in the range of 5–40 glucose residues, with maxima at lengths of 12, 15 and about 21 glucose residues. This pattern was observed regardless of the age and growth conditions of the plants. It may be a common feature of transitory starches: a similar distribution is observed for the amylpectin of the transitory starch of Arabidopsis (Zeeman et al., 1998a).

Increases in the starch content of leaves during the light period are generally accompanied by decreases in the ratio of amylose to amylpectin, and in the molecular mass of amylpectin (Chang, 1979; Matheson, 1996; Tomlinson, 1996). Taken together with the developmental changes in the composition of leaf starches described above, this may indicate that the diurnal changes in starch content occur primarily in an amylpectin fraction of relatively low molecular mass which is preferentially synthesized during the day and preferentially degraded at night. Amylose, and amylpectin of higher molecular mass, appear to accumulate gradually as the leaf ages.
Very little is known about the organization of amylose and amylopectin within granules of transitory starch. In general it appears that these granules lack the relatively high level of crystalline order seen in storage starches (Radwan and Stocking, 1957; Steup et al., 1983). However, like the starch of storage organs, the starch of pea leaves is at least in part semi-crystalline with a lamellar repeat of 9 nm (Waigh, 1997). The starch of tobacco leaves contained amorphous and semi-crystalline zones analogous to the growth rings of storage starches (Buttrose, 1963), and the starch of spinach leaves was reported to consist of an amorphous outer ‘mantle’ and an inner, more crystalline core. The mantle was more readily degraded during the night than the core (Beck, 1985). The mantle may represent the amylopectin of relatively low molecular mass that is preferentially turned over on a diurnal basis. The core, and the material organised into growth rings in tobacco starch, may represent material that accumulates as the basal level of starch in these leaves increases with leaf age. The material in transitory starches that is not turned over on a diurnal basis may thus resemble storage starch in that it contains amylopectin molecules of high molecular mass and considerable amounts of amylose and it is probably semi-crystalline.

IV. The Synthesis of Transitory Starch

A. Pathway

Starch in chloroplasts is synthesized from fructose 6-phosphate derived from the reductive pentose phosphate pathway. Fructose 6-phosphate is converted to glucose 6-phosphate and then to glucose 1-phosphate via the enzymes phosphoglucoisomerase and PGM (Fig. 2). The substrate for the synthesis of the starch polymers, ADPglucose, is synthesized from glucose 1-phosphate and ATP via the enzyme AGPase (EC 2.7.7.27) in a reaction which also produces pyrophosphate (Fig. 3). This reaction is rendered effectively irreversible in vivo by the presence in the chloroplast of an alkaline inorganic pyrophosphatase, which hydrolyzes the pyrophosphate produced to phosphate (Weiner et al., 1987).

Evidence that this pathway is exclusively responsible for starch synthesis in leaves comes from mutant plants with reduced or undetectable activities in leaves of either plastidial PGM or AGPase. Both classes of mutant are either unable to synthesize transitory starch or have a severely restricted capacity to do so. For example, peas carrying a mutation at the rug3 locus (Harrison et al., 1997), tobacco (Hanson and McHale, 1988, see above) and the Arabidopsis mutant TC7 (Caspar et al., 1986) all lack activity of the plastidial isofrom of PGM, and are unable to synthesize transitory starch. Arabidopsis plants carrying mutations at the adg2 and adg1 loci have 5% of wild-type and undetectable activities of AGPase, respectively (Lin et al., 1988b,c). adg1 mutants are unable to make transitory starch, and adg2 mutants accumulate only 40% of the amount of starch in leaves of wild-type plants.

ADPglucose is the substrate for starch synthase, which adds glucose residues to the non-reducing ends of glucan chains via α(1-4) linkages. The α(1-6) linkages that create the branch points in the starch polymers are introduced by starch-branching enzyme (SBE), which transfers linear glucans from the end

Fig. 2. Pathways of starch and sucrose metabolism in leaves. TPT, triose-phosphate translocator.
of a linear chain to the side of the same or an adjacent chain (Fig. 3).

The present understanding of the nature and properties of AGPase, starch synthase and SBE in leaves, and the roles of starch synthase and SBE in determining starch structure, are considered below.

1. ADPglucose Pyrophosphorylase

The activity of AGPase in leaves is potentially subject to regulation both allosterically by metabolites, and at the levels of transcription and translation. Our description will concentrate on aspects of its regulation that are of relevance to its role in the regulation of starch synthesis (Section IV.B).

AGPase from leaves is allosterically regulated by a number of metabolites central to chloroplast metabolism. It is activated most strongly by 3-phosphoglycerate (3-PGA): for example the enzymes from spinach and barley leaves are activated about 25-fold and 13-fold with values of and respectively (Morell et al., 1988; Kleczkowski et al., 1993)—but fructose 6-phosphate, fructose 1,6-bisphosphate, phosphoenolpyruvate and 2-phosphoglycerate also activate the enzyme from some sources (Sanwal et al., 1968). Inorganic phosphate is a potent inhibitor, with values of and for the enzymes from spinach and barley leaves respectively. The presence of 3-PGA weakens and alters the nature of the inhibition by causing the inhibition curve to change from hyperbolic to sigmoidal (Copeland and Preiss, 1981; Kleczkowski et al., 1993). The activity of the enzyme is thus extremely sensitive to changes in the ratio of 3-PGA to P$_i$, and this has important consequences for its regulation in the chloroplast (see Section IV.B).

AGPase is a heterotetramer of two types of subunit—known as large and small subunits—that are related in sequence (Smith-White and Preiss, 1992). The precise roles of the two subunits in determining the properties of the enzyme are not yet fully understood, and both appear to be capable of binding substrates and effectors. Studies of the catalytic properties of large and small subunits expressed separately and together in the bacterium Escherichia coli are consistent with the view that the small subunits form a basic catalytic unit, the regulatory properties of which are modified by association with a large subunit (Ballicora et al., 1995; Preiss and Sivak, 1996).

Many species of plant contain small multigene families encoding one or both of the subunits. The small subunit is evolutionarily highly conserved while the large subunit shows much more divergence of sequence between species, and between members of multigene families within a species. Analysis of transcripts indicates that members of large and small subunit families are differentially expressed between organs, and during the development of individual organs (Krishnan et al., 1986; Olive et al., 1989; Prioul et al., 1994; La Cognata et al., 1995; Weber et al., 1995).

The expression of subunit genes has been studied in most detail in potato. One gene encoding a small subunit and two different genes encoding large subunits are expressed in potato leaves. Transcript

Fig. 3. Pathway of starch synthesis in leaves. PPI, inorganic pyrophosphate; Pi, inorganic phosphate.
from the large-subunit gene \textit{agpS2} was more abundant in young sink leaves than in source leaves, and was weakly induced by incubation of detached leaves in sucrose (La Cognata et al., 1995). Only small amounts of transcript from the second large-subunit gene, \textit{agp5}, were detectable in attached leaves, but levels increased dramatically upon incubation of detached leaves with sucrose (Müller-Röber et al., 1990). The \textit{agp5} and \textit{agpS2} genes appear to be expressed in different tissues within the leaf. A promoter-\(\beta\)-glucuronidase fusion system indicated that the \textit{agpS} gene is expressed in stomata and the starch sheath surrounding the main veins, but apparently not in the mesophyll cells (Müller-Röber et al., 1994). It seems likely that the \textit{agpS2} gene is expressed in mesophyll cells.

Amounts of protein of AGPase subunits in potato leaves do not reflect directly the amounts of transcript, implying that there is strong post-transcriptional regulation of expression. Whereas levels of small-subunit transcript increased during the photoperiod, and levels of both small and large subunit transcripts increased in response to incubation with sucrose, there were no corresponding changes in amounts of the proteins or in AGPase activity (Nakata and Okita, 1995). Activity and amounts stayed constant or actually fell both during the photoperiod and in response to incubation with sucrose.

The significance of this regulatory complexity is not yet clear. It is not known whether the transcripational and post-transcripational regulation of expression actually leads to changes in the subunit composition of AGPase in the mesophyll cells of the leaf during development, on a diurnal basis, or in response to carbohydrate status or environmental conditions. If the subunit composition does change, this may lead to changes in the regulatory properties of the enzyme. It is likely that AGPases with different subunit compositions will be differently sensitive to allosteric activation and inhibition (Ballicora et al., 1995).

AGPase is generally accepted to be an exclusively plastidial enzyme, and there is compelling evidence for some plant organs that this is the case (ap Rees, 1995). However, recent work has demonstrated that endosperms of barley and maize have distinct plastidial and extra-plastidial forms of the enzyme (Shannon et al., 1998; Denyer et al., 1996b; Thorbjørnsen et al., 1996). In both tissues most of the AGPase activity is contributed by the extra-plastidial form, and in maize endosperm analysis of a mutant (\textit{brittle2}) lacking the extraplastidial activity indicates that it provides much of the ADPglucose for starch synthesis in wild-type endosperm (Denyer et al., 1996b; Thorbjørnsen et al., 1996). There is at present no evidence that leaves contain an extra-plastidial AGPase (ap Rees, 1995). In leaves of barley, immunological studies suggested that the AGPase was attributable to chloroplastic proteins likely to be the same as those responsible for the minor, plastidial activity in the endosperm (Thorbjørnsen et al., 1996).

2. Starch Synthase

The starch synthase activity of plant organs is composed of several, distinct isoforms, each encoded by a different gene. Some of these isoforms are soluble, ie in the stroma of the plastid, some are tightly bound to the starch granule, and some are present in both of these locations. For a few starch-synthesising storage organs, most or all of the isoforms have been identified. However, their kinetic and regulatory properties are poorly understood, and in vitro studies have shed little light on their roles in the synthesis of the starch polymers. Recent studies of mutant and transgenic plants with altered isoform compositions are starting to elucidate these roles. Although some of the first descriptions of starch synthases were made on the enzyme from leaves (Frydman and Cardini, 1964; Doi et al., 1965) studies of starch synthase in leaves have been less extensive and less detailed than those in storage organs. We will summarise the present understanding of the enzyme in storage organs, then examine the extent to which this picture is applicable to leaves.

Starch-synthesising storage organs of wild-type plants all contain a highly-conserved class of isoform of starch synthase that is tightly bound to the starch granule. This class is known as granule-bound starch synthase I (GBSSI: Smith et al., 1997). Analysis of mutant and transgenic plants in which GBSSI activity has been reduced or eliminated (for example the \textit{waxy} mutants of cereals, the \textit{lam} mutant of pea, the \textit{amf} mutant of potato, and potatoes expressing antisense RNA for GBSSI: Denyer et al., 1995a and reviewed in Smith and Martin, 1993; Smith et al., 1997) provides evidence consistent with the idea that GBSSI is specifically responsible for the synthesis of the amylose component of the starch (Fig. 3). These mutants have a normal starch content, but the starch consists almost entirely of amylopectin. Exactly how GBSSI is able to synthesise an almost linear glucan
in the same compartment and at the same time as the synthesis of a highly branched glucan is unclear. One possibility is that it is buried within the starch granule in such a way that it is accessible to its substrates—ADPglucose and glucans of low molecular mass—but its product is not accessible to SBEs (Denyer et al., 1993, 1996a). It is also not clear whether GBSSI contributes to the elongation of amylopectin chains in vivo. It is certainly capable of incorporating glucose residues from ADPglucose into long chains of amylopectin in isolated starch granules (Denyer et al., 1996a).

Other isoforms of starch synthase—those responsible for amylopectin synthesis (Fig. 3)—are less well understood. Three classes, distinguished by features of their predicted amino-acid sequences and/or their molecular mass and antigenic properties, have been identified so far. Starch synthase I (SSI) has been identified in potato and the endosperms of rice and maize (Baba et al., 1993; Abel et al., 1996; Knight et al., 1998). SSI in potato, pea embryo and cereal endosperms (Denyer and Smith, 1992; Dry et al., 1992; Denyer et al., 1995b; Edwards et al., 1995; Hylton et al., 1996; Harn et al., 1998), and SSIII in potato, pea embryo and maize endosperm (Abel et al., 1996; Marshall et al., 1996; Craig et al., 1998; Gao et al., 1998). It is likely that these three classes are of widespread occurrence, and between them they may account for starch synthase activity other than that accounted for by GBSSI. However, their relative importances differ from one type of storage organ to another. For example, SSI accounts for 60–70% of the soluble activity in pea embryos (Denyer and Smith, 1992; Edwards et al., 1996), and SSIII was a minor component of the activity (Craig et al., 1998). A mutation that eliminates SSI (at the rug5 locus) dramatically reduces the average chain length of amylopectin in the embryo, indicating that this isoform plays a specific role in determining amylopectin structure (Craig et al., 1998). In contrast, SSI accounts for about 10% and SSIII for 85% of the soluble starch synthase activity in a potato tuber (Edwards et al., 1995; Abel et al., 1996; Marshall et al., 1996). Large reductions in SSI activity in the tuber, achieved through expression of antisense RNA, had very little effect on amylopectin structure (Edwards et al., 1995; Marshall et al., 1996). It is thus not possible at present to make generalizations about the nature and specific roles of isoforms of starch synthase other than GBSSI.

There is good evidence that leaves, like storage organs, contain multiple isoforms of starch synthase, and that these are both soluble and granule bound. Multiple, soluble forms of the enzyme have been resolved by ion-exchange chromatography and gel electrophoresis from spinach, rice and maize leaves (Ozbun et al., 1971, 1972; Hawker et al., 1974; Dang and Boyer, 1988, 1989; Tacke et al., 1991; Yamanouchi and Nakamura, 1992), and granule-bound activity has been reported for starch from, for example, geranium and rice leaves (Frydman and Cardini, 1967; Taira et al., 1991). These reports have generally not identified the proteins actually responsible for the activities, hence it is not possible to determine unequivocally the nature and the number of gene products that contribute to the activity. Nonetheless, there is evidence that the isoforms of starch synthase present in leaves of cereals differ from those in the storage organs of the same plant. For example, Taira et al (1991) reported that the granule-bound activity and the amylose content of the starch of rice leaves were unaffected by the waxy mutation that eliminates GBSSI and amylose from the endosperm. Leaf starch contained no proteins antigenically related to the GBSSI of the endosperm. In maize, the chromatographic behaviour and kinetic properties of the soluble starch synthase activity of leaves and developing endosperm differed considerably, leading to the suggestion that leaves contained only one of the two isoforms distinguished biochemically in endosperm (Dang and Boyer, 1988; 1989).

Detailed study of pea revealed that both the granule-bound and soluble starch synthase activities of leaves are attributable to isoforms different from those in the starch-storing embryo. Leaf starch contains an isoform of starch synthase (GBSSIIb) closely related in kinetic and antigenic properties and in N-terminal sequence to the GBSSI class, but encoded by a different gene from the GBSSI of the embryo (Denyer et al., 1995a, 1997; Tomlinson et al., 1998). Most of the soluble activity in the leaf is contributed by an isoform antigenically related to the SSIII class. Although a minor isoform of the SSIII class is also present in the embryo (Craig et al., 1998) it is not clear whether the leaf and the embryo SSIII-like isoforms are the same gene product. The SSII isoform that contributes 60–70% of the soluble activity of the embryo contributes only about 10% of the soluble activity of the leaf (Tomlinson et al., 1998).

The precise contributions of different isoforms of starch synthase to the synthesis of amylopectin in transitory starches has not been assessed. Very few
isoforms have been positively identified in leaves, and no mutations affecting them have been described. It is nonetheless possible that at least some of the differences in the structure and organization of amyllopectin molecules between transitory and storage starches are caused by differences in the isoform composition of the starch synthases responsible for the synthesis of these two sorts of starch. However, other factors are also likely to contribute to the differences in the structure and organization of amyllopectin molecules. The structure of storage starches is determined over many days, during which time the relative levels of expression of different isoforms of both starch synthase and starch-branching enzyme probably change (Martin and Smith, 1995). Transitory starch, in contrast, is mostly the immediate product of the isoforms of starch synthase and starch branching enzyme present in the chloroplast during the preceding photoperiod. The structure of any starch present at the start of the photoperiod will be strongly influenced by the actions of starch-degrading enzymes during the previous night.

The isoforms of starch synthase responsible for the synthesis of amylose in leaves are not well understood. In potato leaves, amylose synthesis is almost certainly a function of the same GBSSI isoform of starch synthase responsible for amylose synthesis in the tuber. A mutation in the gene encoding GBSSI eliminates amylose from both tuber and leaf starches (Jacobsen et al., 1989). However, as discussed above, leaf amylose is not synthesized by the GBSSI isoform of the storage organ in pea and rice. The lam mutation that eliminates amylose from the starch of pea embryo has no effect on the amylose content of leaf starch. A second isoform of the GBSSI class, GBSSIb, may be responsible for amylose synthesis in pea leaves (Denyer et al., 1997), but definitive proof that this is the case is lacking. It is possible that the amylose of the leaf is at least in part a product of isoforms of starch synthase other than those of the GBSSI class.

3. Starch-Branching Enzyme

Like starch synthase, SBE exists as multiple isoforms that are much better described for storage organs than for leaves. The isoforms of storage organs fall into two distinct classes (known as A and B), distinguished by features of their amino-acid sequences (Burton et al., 1995, Martin and Smith, 1995). All of the storage organs so far examined—including those of rice, pea, maize and potato—possess both A and B isoforms, and these appear to account for all of the activity of SBE in the organ (Smith et al., 1997). In the endosperm of maize, two different A isoforms as well as a B isoform are present (isoforms IIA and IIB: Gao et al., 1997; Boyer and Preiss, 1978a,b) and rice endosperm also appears to contain multiple forms of the two types of isoform (Mizuno et al., 1992, 1993; Yamanouchi and Nakamura, 1992). The A and B isoforms have distinct properties. Detailed studies of these isoforms from maize, both in vitro and when expressed in Escherichia coli, show that the A isoform (maize isoform II) prefers more highly-branched substrates and preferentially transfers shorter branches than the B isoform (maize isoform I; Guan and Preiss, 1993, Takeda et al., 1993; Guan et al., 1995). More limited studies on the A and B isoforms of other species indicate that the properties of the maize isoforms define general differences between the A and B classes (Smith, 1988; Morell et al., 1997).

There is evidence from pea, rice and maize that leaves also contain A and B isoforms. The isoforms of pea leaves are antigenically related to those of the embryo, and the effects of a mutation that eliminates the A isoform (at the r locus: Bhattacharyya et al., 1990; Burton et al., 1995) combined with the results of immunoprecipitation experiments provide good evidence that the same A and B isoforms account for all of the SBE activity of both the leaf and the embryo (Tomlinson et al., 1997). The two main forms of SBE purified from maize and rice leaves have kinetic, antigenic and chromatographic properties similar to the A and B isoforms of the endosperm (Dang and Boyer, 1988; Yamanouchi and Nakamura, 1992) although the relative contributions of the A and B isoforms to the total SBE activity probably differs between leaf and endosperm. Biochemical evidence, and the effects on leaf and endosperm SBE of mutations (at the amyllose-extender loci) that lie in genes encoding an A isoform (maize isoform IIB) indicate that leaves of maize and rice may contain a different A isoform from the endosperms of these species (Boyer and Preiss, 1978a,b; Dang and Boyer, 1988, 1989; Yamanouchi and Nakamura, 1992; Mizuno et al., 1993; Fisher et al., 1996; Gao et al., 1996).

The fact that starch-synthesising organs have two isoforms of SBE with different properties has led to speculation that the two isoforms create branches of
different lengths or at different intervals along the amylopectin chains during amylopectin synthesis in vivo. For example, it has been suggested that the A isoform creates the chains of 12–16 glucose units that lie within the amylopectin clusters and the B isoform creates the longer chains that span clusters (Takeda et al., 1993; Preiss and Sivak, 1996). Studies of the effects of the r mutation that eliminates the A isoform in peas (Bhattacharyya et al., 1990) show that this idea requires refinement. Although the mutation increases the average chain length of amylopectin in both embryos and leaves, it has little effect on the polymodal distribution of chain lengths. The amylopectin of mutant leaves—containing only the B isoform—has a distribution of chain lengths from 5 to 40 glucose that is virtually indistinguishable from the wild-type (Tomlinson et al., 1997). The extent to which the A and B isoforms play distinct roles in amylopectin synthesis in vivo remains unclear.

4. Debranching enzyme

Studies of mutants of cereals and *Chlamydomonas* that are deficient in debranching enzyme activity have given rise to the proposal that these enzymes, as well as SBEs, are important in determining the branching pattern of amylopectin. The developing endosperms of *sugary1* maize and rice accumulate a highly-branched, water-soluble glucan, phytoglycogen, in place of much of their starch. Both endosperms have reduced activities of debranching enzyme, and the gene at the sugary1 (su1) locus of maize has been shown to encode a debranching enzyme of the isoamylase class (Pan and Nelson, 1984; Doehlert et al., 1993, James et al., 1995, Nakamura et al., 1996). Mutations at the sta7 locus of *Chlamydomonas* cause the production of phytoglycogen instead of starch, and are associated with the loss of a debranching enzyme (Mouille et al., 1996). It is proposed on the basis of these phenotypes that debranching enzyme may act together with starch-branching enzyme to determine the degree of branching of amylopectin in a broad range of starch-synthesising species. Ball and colleagues (Ball et al., 1996) put forward a model for the synthesis of amylopectin in which trimming by debranching enzyme of a highly-branched, ‘pre-amylopectin’, synthesized by starch synthases and SBEs, is an essential step. It has proved difficult to test the model rigorously, in part because of lack of detailed knowledge of the occurrence and properties of debranching enzymes and in part because the su1 mutations have multiple pleiotropic effects on enzymes of starch synthesis in the endosperm. Although the mutation in maize lies in an isoamylase gene, the mutant endosperm has lower activity of a second class of debranching enzyme, pullulanase (also called limit-dextrinase or R enzyme), and alterations in activities of several other enzymes (Doehlert et al., 1993; Singletary et al., 1997) It is thus not possible to attribute the accumulation of phytoglycogen in endosperms unambiguously to the loss of isoamylase activity.

New light has been shed on the involvement of isoamylase in starch synthesis in general, and in starch synthesis in leaves in particular, by the discovery of an *Arabidopsis* mutant that contains phytoglycogen as well as starch in its leaves. This mutant (*dbel*) is analogous to the sul mutants in that isoamylase activity is dramatically reduced or absent in its leaves, and there is evidence that the mutation lies in an isoamylase gene (Zeeman et al., 1998a). Unlike the su1 mutants, the *dbel* mutant is not deficient in pullulanase activity, and there are no major effects on other enzymes of starch synthesis. Phytoglycogen accumulates in mutant leaves at the same time and in the same chloroplasts as starch containing amylopectin of essentially identical structure to that in wild-type leaves (Zeeman et al., 1998a).

This phenotype is not obviously compatible with the trimming model proposed by Ball and colleagues. The simplest expectation from the model is that lack of debranching enzyme would give phytoglycogen only, and reduced debranching activity would give a single type of polymer with a structure intermediate between that of phytoglycogen and amylopectin. To account for the simultaneous accumulation of phytoglycogen and amylopectin in chloroplasts of the *dbel* mutant, Zeeman et al. (1998a) propose that isoamylase is not directly involved in amylopectin synthesis, but rather plays a role in preventing elaboration by starch synthase and SBE of small, soluble glucans in the stroma. They argue that such glucans—for example maltose and maltotriose—can potentially be elongated by starch synthase and then branched by SBE. In the normal plastid, this elaboration of soluble glucan is prevented by a suite of stromal starch-metabolising enzymes such as amylases, phosphorylase and isoamylase. In an isoamylase-deficient mutant, branched soluble glucan can be synthesized in the stroma. Its synthesis
proceeds primarily through changes in the 3-PGA to Pi ratio. First, there are good theoretical reasons for believing that the ratio of 3-PGA to Pi in the chloroplast will change as the ratio of assimilation to sucrose synthesis changes. When the rate of assimilation exceeds the rate of sucrose synthesis, import of Pi and export of triose phosphate from the chloroplast via the TPT is expected to be restricted, leading to an increase in the 3-PGA to Pi ratio in the chloroplast. Conversely, when the rate of assimilation is lower than the rate of sucrose synthesis then the high rate of generation of Pi in the cytosol from sucrose synthesis is expected to result in an increased export of triose phosphate from the chloroplast and a relatively low 3-PGA to Pi ratio in the stroma (Stitt and Quick, 1989). Reports of stromal phosphate concentrations, particularly the reduction in stromal phosphate concentration observed following feedback limitation of photosynthesis (Sharkey and Vanderveer, 1989) are consistent with these models. Further support is provided by the strong evidence that signal metabolite fructose 2,6-bisphosphate is involved in linking the biosynthesis of sucrose in the cytosol to the 3-PGA to Pi ratio in the chloroplast (see Stitt, 1990 for a review). This is discussed in accompanying chapters. We wish only to highlight the link between sucrose sufficiency and the 3-PGA to Pi ratio. Studies of spinach leaves show that towards the end of the photoperiod, when the assimilation rate exceeds the rate of sucrose biosynthesis, there is a restriction in sucrose-phosphate synthase activity and a consequent rise in the hexose phosphate levels (Gerhardt et al., 1987). The rise in fructose 6-phosphate stimulates fructose 6-phosphate 2-kinase and inhibits fructose 2,6-bisphosphatase (Stitt, 1990). The subsequent increase in fructose 2,6-bisphosphate inhibits fructose 1,6-bisphosphatase, which in turn leads to an increase in 3-PGA and triose phosphate in the cytosol. The increased concentration of phosphorylated compounds in the cytosol probably leads to a restriction in the availability of Pi in the chloroplast (Stitt and Quick, 1989). When Pi becomes limiting in the chloroplast, the stromal ATP concentration falls, and the rate of reduction of 3-PGA to triose phosphate is decreased (Heldt et al., 1977; Walker and Sivak, 1986), thus leading to a rise in the stromal 3-PGA to Pi ratio and a stimulation of starch synthesis. Recently, further evidence for the importance of fructose 2,6-bisphosphate has come from studies with transgenic tobacco plants that contain elevated levels of this signal metabolite (Scott et al., 1995; Scott and Kruger, 1995). The authors
found an increased partitioning of carbohydrate towards starch in plants with elevated fructose 2,6-bisphosphate levels and proposed that this switch in partitioning was mediated through the 3-PGA to $P_i$ ratio.

Second, studies with isolated chloroplasts confirm that changes in the ratio of 3-PGA to $P_i$ can alter the rate of starch synthesis. A decrease in the supply of $P_i$ to isolated chloroplasts caused an increase in the partitioning of assimilated carbon into starch. It was proposed that the low concentration of $P_i$ led to a decrease in the ATP/ADP ratio and hence to a restriction of 3-PGA reduction. An increase in the 3-PGA to $P_i$ ratio in the chloroplast would then lead to an activation of AGPase and starch synthesis (Heldt et al., 1977; Robinson and Walker, 1979).

Third, circumstantial evidence for the importance of the 3-PGA to $P_i$ ratio in regulating starch synthesis in vivo has come from studies where the activity of the TPT has been altered through genetic manipulation. The TPT is the major protein in the chloroplast inner envelope and catalyzes a strict 1:1 counter-exchange of substrates: it has similar affinities for triose phosphates, 3-PGA and $P_i$ (Fliege et al., 1978; Flügge and Heldt, 1991). It therefore plays a central role in the regulation of the 3-PGA to $P_i$ ratio and the communication of this ratio between cytosol and stroma. Transgenic lines of potato (Riesmeier et al., 1993) and tobacco (Barnes et al., 1994) have been generated in which TPT activity is reduced through the expression of antisense RNA. A 20–30% reduction in TPT activity in potato led to an increase in leaf starch by a factor of 300% while in tobacco an 80% reduction in activity led to an increase in leaf starch of only 10%. In both cases it would be expected that restricted TPT activity would increase the stromal 3-PGA to $P_i$ ratio and hence stimulate starch synthesis. The fact that the extent of the increase in starch in response to a decrease in TPT activity is different in potato and tobacco indicates that factors other than simply the 3-PGA to $P_i$ ratio are likely to be involved in regulating starch synthesis in some species.

Fourth, the importance of the AGPase in determining the rate of starch synthesis has been clearly shown by mutant and transgenic plants in which the activity of AGPase is reduced. A reduction of 50% in AGPase activity in a mutant Arabidopsis led to 23 and 39% reductions in starch synthesis under low and high light, respectively (Neuhaus and Stitt, 1990). Antisense inhibition of AGPase in potato using a leaf-specific promoter revealed a good positive correlation between the AGPase activity and the accumulation of starch in the leaf (Leidreiter et al., 1995).

The case for the modulation of starch synthesis through the allosteric regulation of AGPase by the 3-PGA to $P_i$ ratio is convincing. However, it should not be assumed that the AGPase is the only site at which control is exerted over the rate of starch synthesis. The development of a sound theoretical approach to the analysis of metabolic fluxes (Kacser and Burns, 1973; ap Rees and Hill, 1994; Kacser et al., 1995) has led to an increased realization that every step in an enzymic sequence contributes to some extent to the determination of flux. The role of each enzyme in controlling flux can be summarised as a flux control coefficient which is the fractional change in flux given by a fractional change in this enzyme activity. The larger the flux control coefficient of a particular enzyme, the more important this enzyme is in controlling the flux. By definition the flux control coefficients of a linear sequence of reactions without branch points sum to 1. It should therefore be the aim of a biochemist to determine all the flux control coefficients in a given pathway, although such an approach is beset by practical problems and has not yet been achieved for an entire pathway in plants (ap Rees and Hill, 1994). In the case of transitory starch synthesis some initial estimates of flux control coefficients have been made using data from a pea mutant deficient in starch-branching enzyme, Arabidopsis mutants deficient in plastidial PGM and AGPase, and a Clarkia xantiana mutant defective in plastidial phosphoglucoisomerase (Kruckeberg et al., 1989; Neuhaus et al., 1989; Neuhaus and Stitt, 1990; Smith et al., 1990). The calculated flux control coefficients indicate that AGPase dominates the control of starch synthesis in both high and low light. The flux control coefficients of phosphoglucoisomerase, PGM and SBE in high light were significantly greater than in low light. This analysis must be regarded as preliminary and incomplete because it is an amalgamation of data from different species and does not include an estimate of the flux control coefficients for the starch synthases. However, it is valuable in indicating that the balance of control in the pathway can be different according to the environmental conditions. Despite the technical problems associated with the determination of flux control coefficients in plants, it is to be hoped that further progress will be made in this direction in the future.
V. The Degradation of Transitory Starch

A. Enzymes Implicated in the Degradation of Transitory Starch

Despite extensive investigation, the precise nature of the pathway of transitory starch degradation remains unclear. Many enzyme activities that could contribute to degradative pathways have been described and characterised, and cDNAs encoding some of these enzymes have been cloned. However, the precise function of many of degradative enzymes in vivo is not known, and in several cases it is not clear whether the degradative activities are localised in the chloroplast. The possible roles of the degradative enzymes in starch degradation are summarised in Fig. 4. In this section, the enzymes will be described and evidence that they may be involved in transitory starch degradation will be discussed.

1. Amylase

Amylases are widespread in higher plants. They can be divided into two types: endoamylases which can catalyze the hydrolysis of the $\alpha$(1-4) linkages at any point within amylose and amylopectin except immediately adjacent to $\alpha$(1-6) branch points, and exoamylases which cleave maltose groups only from the non-reducing end of $\alpha$(1-4)glucan chains. In plants the endoamylase and exoamylase activities are normally called $\alpha$-amylase (EC 3.2.1.1) and $\beta$-amylase (EC 3.2.1.2), respectively.

a. $\alpha$-amylase

Multiple isoforms of $\alpha$-amylase are present in the leaves of many species of plant. Although at least some of these isoforms are not present in the chloroplast, others are chloroplastic and could therefore be involved in transitory starch degradation. Leaves of sugar beet, for example, contain five forms of $\alpha$-amylase separable on zymograms, of which one is extrachloroplastic and four are exclusively or at least partly chloroplastic (Li et al., 1992a). Reports of the nature of the chloroplastic $\alpha$-amylases differ from one species to another. Those of mesophyll and bundle-sheath cells of pearl millet (Pennisetum americanum) leaves (Vally and Sharma, 1995) are reported to be of the heat-stable, calcium-dependent type extensively characterised from cereal endosperms (Briggs, 1967). Calcium-dependent $\alpha$-amylases have also been reported from the leaves of sugar beet (Li et al., 1992a) and poplar (Witt and Sauter, 1996), but the enzyme was extrachloroplastic in the former case and localization studies were not performed in the latter case. The $\alpha$-amylase of spinach chloroplasts, in contrast, is reported to be of the heat-labile, calcium-independent type first described from Arum spadix (Okita et al., 1979; Bulpin and ap Rees, 1978). Activity of $\alpha$-amylase was not detected in studies of the chloroplasts of pea (Stitt et al., 1978) and barley (Jacobsen et al., 1986) leaves. However, in these cases the assays were conducted under conditions in which only heat-stable, calcium dependent $\alpha$-amylases would be detected. The possibility that heat-labile, calcium-independent $\alpha$-amylases are of widespread occurrence in chloroplasts requires further investigation.

There are two recent reports of amylases from Arabidopsis leaves which may be involved in chloroplastic starch degradation. First, activity of an isoform of endoamylase with a pH optimum of 7 is reported to vary diurnally in a manner consistent...
with a role in starch degradation at night (Kakefuda and Preiss, 1997). However, it has not been established whether this activity is in the chloroplast. Second, evidence for the involvement of a different endoamylase in starch degradation has been provided by the discovery of a mutant (sex4) which apparently specifically lacks one isoform of the enzyme. The mutant is deficient in starch degradation at night (Zeeman et al., 1998b). Although this endoamylase has been shown to be chloroplastic, its activity does not vary on a diurnal basis. The possible roles of these two endoamylases are discussed further in Sections VB and VC below.

b. β-amylase

β-amylase is present in leaves, and is often discussed in the context of starch degradation in plants. However, it now seems unlikely that this enzyme plays a role in degradation of transitory starch. Rigorously-conducted cell-fractionation studies have shown that the enzyme is not associated with the chloroplasts in pea leaves (Stitt et al., 1978), and that it is associated with the vacuole rather than the chloroplast in protoplasts derived from pea and wheat leaves (Ziegler and Beck, 1986). A report that β-amylase is present in pea chloroplasts (Kakefuda et al., 1986) may be based on preparations of chloroplasts with significant vacuolar contamination. Extensive studies of the β-amylase of Arabidopsis leaves—where this enzyme accounts for over 80% of the total amylolytic activity—suggest that it is not chloroplastic but is likely to be vacuolar and perhaps localised within the vascular bundle (Lin et al., 1988a; Munroe et al., 1991; Wang et al., 1995).

2. Debranching enzyme

Debranching enzymes catalyze the hydrolysis of α(1–6) linkages in starch. Two classes of these enzymes have been described from higher plants. Pullulanase [also called limit dextrinase or R-enzyme: amylopectin 6–glucanohydrolase (EC 3.2.1.41)] can hydrolyze the yeast glucan pullulan in addition to starch polymers, whereas isoamylase (EC 3.2.1.68) cannot. Pullulanases have been shown to be present in the leaves of several species of plants (sugar beet, Li et al., 1992a; pea, Kakefuda et al., 1986; spinach, Ludwig et al., 1984, Vicia faba: Ghiena et al., 1993). Both extrachloroplastic and chloroplastic isoforms occur in pea leaves (Kakefuda et al., 1986), and chloroplasts of spinach (Ludwig et al., 1984) and Vicia faba (Ghiena et al., 1993) contain pullulanase activity. Isoamylases have been reported from kernels of maize (Manners and Rowe, 1969; Doehlert and Knutson, 1991), pea embryos (Zhu et al., 1998), leaves of Arabidopsis (Zeeman et al., 1998a) and potato tubers (Ishizaki et al., 1978, 1983). Possible roles for debranching enzymes in the synthesis of starch are discussed in Section IVA.4 above.

3. α-glucosidase

α-glucosidase (EC 3.2.1.20), also called maltase, hydrolyzes maltose and malto-oligosaccharides to glucose. α-glucosidases often display wide substrate preferences, which may differ considerably from one source of the enzyme to another. For example, some are specific for α(1–4) linkages and short malto-oligosaccharides, whereas others will hydrolyze α(1–6) and other α-glucosidic linkages in larger substrates. Thus, depending on their characteristics, these enzymes could act solely on the products of amylolytic degradation of starch to ensure their complete conversion to glucose, or could play a wider role in starch degradation by attacking larger, α(1–4), α(1–6) glucans.

There are conflicting reports on the occurrence and subcellular localization of α-glucosidase in leaves. Activity was not detected in studies of pea leaves (Kakefuda et al., 1986) and chloroplasts of pea (Kruger and ap Rees, 1983a) and spinach (Okita et al., 1979). However, Beers et al. (1990) identified two apoplastic and one chloroplastic isoform from pea seedlings. The chloroplastic isoform had a pH optimum of 7.0 and a K_m for maltose of 7.2 mM (Sun et al., 1995). Beers and colleagues suggested that failure to detect activity in previous studies was due to inappropriate extraction and assay conditions. The potential contribution of this enzyme to starch degradation in leaves generally thus remains to be established.

4. Maltose Phosphorylase

Little is known about the occurrence of maltose phosphorylase (EC 2.4.1.8) in higher plants. The enzyme is reported to be present in pea chloroplasts (Kruger and ap Rees, 1983b), but we know of no other information on its occurrence in leaves. Maltose phosphorylase catalyzes the interconversion of maltose with glucose 1-phosphate and glucose, and
could potentially contribute to the conversion to sugars of the products of amylolytic degradation of starch.

5. Starch Phosphorylase

Starch phosphorylase (α-glucan phosphorylase, EC 2.4.1.1) is of wide occurrence in higher plants. The enzyme catalyzes the phosphorolytic cleavage of a glucose residue from the non-reducing end of a glucan chain, to form glucose 1-phosphate. Plastidial and extraplastidial isoforms of the enzyme (referred to as type I and type II phosphorylases) have been demonstrated in many plant organs, including leaves. The properties and primary sequences of the two types are distinct and highly conserved (Steup and Schächtele, 1981; Fukui et al., 1987; Steup, 1988; Duwenig et al., 1998). In potato, in which the enzyme has been very extensively studied (Shimomura et al., 1982; Brisson et al., 1989; Nakano et al., 1989; Steup, 1990; Mori et al., 1991; Sonnewald et al., 1995), there are two plastidial isoforms, encoded by the pho1a and pho1b genes. The Phola product accounts for most of the plastidial activity in the tuber, and the Pho1b product for most of the activity in chloroplasts. Two plastidial isoforms have also been identified in pea (Steup and Latzko, 1979).

Plastidial isoforms of phosphorylase, including those of chloroplasts, prefer malto-oligosaccharides to larger, branched glucans as substrates (Fukui et al., 1987; Steup, 1988). The smallest malto-oligosaccharide that will act as a substrate is maltotetraose (Steup and Schächtele, 1981). There is reason to believe that the reaction catalyzed by the enzyme in the chloroplast is effectively irreversible in the direction of glucose 1-phosphate synthesis. Kruger and ap Rees (1983b) found that the apparent equilibrium constant (0.3–0.7) was very different from the mass action ratio of glucose 1-phosphate:P i in the chloroplast (7.9 × 10^-3) calculated from estimates of stromal volume and metabolite contents in spinach leaves (Wirtz et al., 1980). These properties are consistent with a potential role for the enzyme in the conversion to hexose phosphate of the products of amylolytic degradation of starch. Partially-purified starch phosphorylase from pea chloroplasts exhibits some regulatory properties that could be of relevance to a role in the degradation of transitory starch at night. Its activity is twice as great at pH 7.2 as at pH 8.0, and it is inhibited by 60% in the presence of 4 mM ADPglucose (Kruger and ap Rees, 1983b).

6. D-Enzyme

D-enzyme (4-α-D-glucanotransferase; EC 2.4.1.25) catalyzes a ‘disproportionating’ reaction:

\[
\alpha(1-4)\text{glucan}_{(a)} + \alpha(1-4)\text{glucan}_{(b)} \rightarrow \alpha(1-4)\text{glucan}_{(a+b)} + \text{glucose}
\]

The enzyme has been studied in relatively few plant organs, but has been shown to be chloroplastic in pea (Kakefuda et al., 1986) and Arabidopsis (Lin and Preiss, 1988) leaves. The cDNA for D-enzyme from potato tubers encodes a putative plastid transit peptide (Takaha et al., 1993). D-enzyme could thus potentially convert short glucans generated by degradation of transitory starch into longer substrates more suitable for amylases and phosphorylase. A recent study of the properties of a D-enzyme from potato following its expression in E. coli reveals that the enzyme can also catalyze the formation of cyclic glucans from amylose (Takaha et al., 1996). The existence of such cyclic glucans in plants has not been demonstrated. Large reductions in the activity of D-enzyme brought about by the expression of antisense RNA in potatoes reduced the growth rate of the plant (Takaha et al., 1998), but the precise role played by the enzyme in the metabolism of starch in vivo remains to be established.

7. Hexokinase

The entry into intermediary metabolism of glucose released from starch requires the formation of glucose 6-phosphate via a hexokinase (EC 2.7.1.1). There is continuing debate about whether this phosphorylation occurs within the chloroplast or in the cytosol. The ability of chloroplasts from several species of plant, including pea and spinach, to metabolise exogenously-supplied glucose suggests a chloroplastic location for hexokinase (Stitt, 1984). However, cell-fractionation experiments indicated that hexokinase activity is outside the chloroplast envelope in pea and spinach (Stitt, 1984; Schnarrenberger, 1990). It seems likely that hexokinase is actually located on the chloroplast envelope, but definitive proof that this is the case is lacking.

8. R1 Protein

A novel protein implicated in the process of starch degradation in leaves has recently been identified in
potato (Lorbeth et al., 1998). The protein (named R1) was first identified from the matrix of tuber starch granules. An immuno-screening approach led to the isolation of cDNA encoding a large protein of 1464 amino acids with no obvious sequence similarity to any other known protein. No enzymatic activity could be assigned to the protein expressed from the cDNA. Expression of antisense RNA for R1 in potatoes had several important effects on the nature and content of starch in the plant. First, there was a reduction in the phosphate content of tuber starch although the amount of starch in the tuber was not affected. Second, leaves contained more starch than those of untransformed controls: complete mobilization of starch during extended periods of darkness was not achieved. Third, a significant reduction in cold sweetening in cold-stored tubers was found in conjunction with an enhanced retention of starch after two months of storage. It is difficult to deduce the precise role of R1 from these complex phenotypic effects. However, expression of the R1 protein in E. coli led to a significant increase in the level of phosphorylation of the glycogen made by the bacterium. The authors proposed that the primary function of R1 is in starch phosphorylation, and that a lowering of the phosphate content of the starch of the transgenic potatoes leads by an unknown secondary mechanism to reduced susceptibility to degradation (Lorbeth et al., 1998). Further investigation, including the development of an assay, will be required to resolve the role of R1.

B. The Pathway of Transitory Starch Degradation in vivo

There is considerable confusion over which of the above enzymes are important in the degradation of transitory starch. The enzymes can be grouped into pathways around a hydrolytic or a phosphorolytic cleavage of \( \alpha(1-4) \) linkages, but it is not clear whether one of these two pathways operates in vivo or whether both occur simultaneously. We shall consider the evidence available for transitory starch about the nature of the initial attack on the starch granule, and the fate of products of this initial attack.

There is some evidence that both starch phosphorylase and hydrolytic enzymes may be able to attack intact starch granules from leaves. Endoamylases from several sources and a putative chloroplastic \( \alpha \)-glucosidase from pea leaves have been reported to attack isolated starch granules (Stitt and Steup, 1985; Li et al., 1992a; Sun et al., 1995; Witt and Sauter, 1995). Starch phosphorylase from pea chloroplasts (Kruger and ap Rees, 1983b) and poplar wood (Witt and Sauter, 1995) was also reported to attack intact starch granules, but Steup et al. (1983) were unable to demonstrate degradation of starch granules by this enzyme. All of these in vitro experiments on the degradation of starch granules must be interpreted with caution since they are prone to several problems. First, the properties of the degradative enzymes may be altered during purification. Second, the properties of the starch granules may be altered during isolation. In particular, newly-synthesized, less crystalline material—which would be the substrate for degradative enzymes in vivo—may be lost. Third, some studies have used granules from a different plant source from the degradative enzymes, or have not taken care to isolate granules at the end of a photoperiod when degradation normally commences in vivo. Finally, the possibility that isolated granules may be contaminated with degradative enzymes has not been considered in all studies.

The only direct evidence for the involvement of a specific starch-degrading enzyme in starch degradation in leaves comes from studies of the sex4 mutant of Arabidopsis. This mutant is deficient in starch degradation at night. Although some degradation occurs, the rate is much lower than in wild-type leaves, and as a result starch accumulates to high levels as the leaf develops (Zeeman et al., 1998b). The mutant lacks one, chloroplastic isofrom of endoamylase (termed A2), identified by native gel electrophoresis of extracts of wild-type leaves. Other enzymes of starch synthesis and degradation, including debranching enzymes, phosphorylase and amylases, are apparently unaffected by the mutation. Although the gene at the sex4 locus has not been identified, it is likely that the reduced rate of starch degradation in sex4 leaves is directly attributable to the loss of the A2 endoamylase. The properties of this enzyme have not yet been established.

Studies with isolated chloroplasts have provided some evidence that the hydrolytic and phosphorolytic pathways can both operate in the degradation of transitory starch. Stitt and ap Rees (1980) labeled the starch in chloroplasts isolated from pea shoots by incubating the chloroplasts with \(^{14}\text{C-glucose} \) in the light. During the subsequent dark period, \(^{14}\text{C} \) lost from the starch appeared predominantly in maltose but also in sugar phosphates and 3-PGA. The situation
was similar in experiments with chloroplasts isolated from spinach leaves: $^{14}$C lost during degradation of $^{14}$C starch was found in glucose, maltose, 3-PGA, triose phosphates and $\text{CO}_2$ (Stitt and Heldt, 1981). The conclusion that both phosphorolytic and hydrolytic pathways are operating in these chloroplasts must, however, be treated with caution. It is theoretically possible that the products of apparent hydrolytic degradation are in fact synthesized from the products of phosphorolytic degradation (Kruger and ap Rees 1983a). In experiments with pea chloroplasts, the presence of maltose phosphorylase and the dependence of maltose synthesis on exogenous $\text{P}_i$ led to the suggestion that maltose was synthesized from glucose 1-phosphate formed via phosphorolytic degradation of starch (Kruger and ap Rees, 1983a). Taken as a whole, therefore, experiments with isolated starch granules and chloroplasts have thus far failed to define the pathway of starch degradation.

C. Regulation of the Degradation of Transitory Starch

Given the uncertainty over the pathway of starch degradation, it is impossible to draw any firm conclusions about the regulation of the process. Net degradation occurs predominantly during the night, although some studies have reported that net starch degradation can also occur at the beginning and end of the photoperiod (spinach, Servaites et al., 1989; sugar beet and Phaseolus vulgaris, Fondy et al., 1989). One possible model is that the rate of starch degradation in leaves is constant, and that changes in the amount of starch are brought about solely by changes in the rate of synthesis (Preiss, 1982). However, observations about the timing and rate of degradation of transitory starch indicate this is not the case, and that the process must be subject to tight regulation. First, radioactive labeling experiments have provided no evidence for starch turnover in illuminated leaves during the normal photoperiod (pea, Kruger et al., 1983; sugar beet, Li et al., 1992). Second, the rate of degradation is subject to variation during the dark period. Whereas degradation of transitory starch can occur immediately following the light/dark transition in some species (e.g., pea, Stitt et al., 1978), in others there is a lag at the beginning of the dark period before degradation commences. In studies of spinach (Stitt et al., 1978) and soybean (Mullen and Koller, 1988a) leaves, sucrose content started to fall immediately after the light/dark transition but there was a short lag before starch degradation occurred. In barley leaves (Gordon et al., 1980), starch degradation did not occur until sucrose content fell below a threshold value, nine hours after the start of the dark period. A correlation between the starch content of the leaf and the rate of degradation in the dark has been observed in studies of soybeans grown under different light intensities (Mullen and Koller, 1988b), and mutant Arabidopsis lines with differing starch contents (mutants with reduced AGPase, Lin et al., 1988b,c). Taken as a whole these experiments suggest that the rate of starch degradation in leaves may be sensitive to the diurnal cycle, the starch content, and the demand for sucrose in the dark.

The only obvious point at which starch degradation could be regulated is at the reaction catalyzed by starch phosphorylase. The pH optimum, the sensitivity to inhibition by ADPglucose and the dependence upon $\text{P}_i$ of starch phosphorylase would be expected to result in an increase in its activity after a light-dark transition, when stromal pH becomes more alkaline, $\text{P}_i$ concentration probably rises and the ADPglucose concentration falls (Kruger and ap Rees, 1983b). The potential importance of the effects of $\text{P}_i$ concentration on the activity of starch phosphorylase is demonstrated by experiments with isolated chloroplasts, in which the rate of phosphorolytic starch degradation is sensitive to the exogenous $\text{P}_i$ concentration (pea, Stitt and ap Rees, 1980; Kruger and ap Rees, 1983b; spinach, Stitt and Heldt, 1981). The dependence of starch phosphorylase activity on $\text{P}_i$ concentration provides a means by which starch degradation in the leaf may be linked to the demand for sucrose. It is believed that the fall in sucrose content at the onset of the dark period leads to an increase in cytosolic and stromal $\text{P}_i$ concentration. This would be expected to lead to a higher activity of starch phosphorylase, and hence a greater rate of starch degradation (Stitt et al., 1985).

Although the effects of $\text{P}_i$ concentration on starch phosphorylase may contribute to the regulation of starch degradation, it is clear that other mechanisms must also operate. First, although the activity of starch phosphorylase in the chloroplasts of some species is sufficient to account for the rate of starch degradation (e.g., pea, Stitt et al., 1978), in others it is not. In Arabidopsis leaves, only 4% of the starch phosphorylase activity is plastidial, and this plastidial activity is probably lower than the rate of starch degradation.
degradation in the dark (Lin et al., 1988a). Second, removal of exogenous P from isolated spinach chloroplasts in the dark results in a shift from phosphorolytic to apparently amylolytic starch degradation and has little effect on the total rate of degradation (Stitt and Heldt, 1981). Third, a regulatory mechanism based on changes that occur during a light dark transition cannot explain those circumstances in which starch is degraded in the light.

It seems probable that amylolytic enzymes as well as starch phosphorylase must play a role in the regulation of transitory starch degradation. As discussed above, there is good evidence that amylolytic activities occur inside chloroplasts and that, at least in Arabidopsis, they are required for starch degradation. However, apart from a sharp pH optimum around pH 6 (Beck and Ziegler, 1989), there are few reports of properties of chloroplastic amylases that suggest how their activity might be modulated in vivo. There are reports that the total amylolytic activity in chloroplasts of spinach (Pongratz and Beck, 1978) and Vicia faba (Ghiena et al., 1993) is subject to diurnal variation, but these measurements must be treated with caution because the possibility of diurnal variation in the recovery of activity during the extraction procedure was not ruled out. The extractable activity of a specific isoform of endoamylase with a pH optimum of 7 has recently been shown to vary on a diurnal basis in Arabidopsis leaves (Section VA.1). Activity - detected on native gels - was seen only in leaves harvested in the dark, whereas activity of other starch-hydrolysing enzymes did not vary on a diurnal basis (Kakefuda and Preiss, 1997). This amylase has not been shown to be chloroplastic, and its involvement in starch degradation remains to be established.

D. Export of the Products of Starch Degradation from Chloroplasts

The synthesis of sucrose is by far the major fate for the carbon released by degradation of transitory starch. For example, in sugar beet leaves the rates of starch breakdown and sucrose synthesis at night have been estimated as 0.23 and 0.16 μg C cm⁻² per min respectively (Fondy and Geiger, 1982). Transitory starch degradation at night must be precisely integrated with cytosolic metabolism, and in particular with sucrose biosynthesis. The nature of the export mechanisms for carbon from the chloroplast are of central importance to this integration. Recent experiments indicate that the triose phosphate translocator (TPT)—responsible for the export of carbon from the chloroplast during the day—is not the main route of export of carbon produced by the degradation of transitory starch at night. Reduction of the activity of the TPT through the expression of antisense RNA in potato plants results, as expected, in increased accumulation of transitory starch during the day, but also causes increased mobilization of transitory starch and export of sucrose from the leaves at night (Riesmeier et al., 1993, Heineke et al., 1994). In tobacco plants with reduced activities of the TPT, starch degradation is promoted not only at night but also at the end of the light period. Although leaves of transgenic plants contained three times more starch than those of control plants after six hours of the light period, the starch content of the transgenic leaves actually fell towards the end of the light period and transgenic and control leaves contained similarly low amounts of starch by the end of the dark period (Häusler et al., 1998). It seems very unlikely from these results that the products of starch degradation are exported via the TPT.

The results described above strongly indicate that sucrose synthesis at night is supported by export from the chloroplast of metabolites other than triose phosphate. Three further observations lend support to this idea. First, mutants of Flaveria linearis with very low activities of cytosolic fructose bisphosphatase (Sharkey et al., 1992) and transgenic potato plants with large reductions in cytosolic fructose bisphosphatase brought about by the expression of antisense RNA (Zrenner et al., 1996) partitioned more of their photosynthate into starch and less into sucrose during the day than did wild-type plants. This result is consistent with a restricted ability to convert triose phosphate to sucrose in the mutant and transgenic plants. However, during the night the rate of starch mobilization in leaves of mutant and transgenic plants was twice as great as in wild-type plants, indicating that carbon exported from the chloroplast at night was not in the form of triose phosphates. Second, radioactive feeding experiments with wild-type Arabidopsis leaves showed that there is a net glycolytic flux at night: if carbon export from the chloroplast were at the level of triose phosphates there would have to be a net gluconeogenic flux (Trethewey et al., 1994b). Third, leaves of several species have a high concentration of fructose 2,6-bisphosphate at night (Stitt et al., 1985; Usuda et al.,
This should ensure that the cytosolic fructose 1,6-bisphosphatase is substantially inhibited, and thus prevent a gluconeogenic flux from triose phosphate to sucrose. These experiments suggest strongly that carbon for sucrose synthesis must be exported from the chloroplast as a sugar or a sugar phosphate.

A direct demonstration of a TPT-independent route for export from the chloroplast has been provided by investigations into an *Arabidopsis* mutant, *sex1*, that has severely reduced rates of starch degradation (TC265; Caspar et al., 1991; Trethewey and ap Rees, 1994a,b). The mutation results in a four- to five-fold accumulation of starch, and a reduced growth rate. The mutant contained appreciable activities of all of the enzymes putatively involved in starch degradation (Section V.A; Caspar et al., 1991; R. Trethewey, unpublished), but physiological analysis clearly indicated that it was defective in the conversion of starch to sucrose at night (Trethewey and ap Rees, 1994a,b). Uptake studies on intact chloroplasts isolated from mutant and wild type plants revealed that the mutant was deficient in glucose uptake (Trethewey et al., 1994a). This work suggested strongly that the products of starch degradation in *Arabidopsis* chloroplasts are exported via a glucose transporter. Evidence that this may also be the case in tobacco leaves comes from transgenic plants with reduced activity of the TPT. As described above, these plants show increased rates of starch degradation at night and at the end of the light period. This phenomenon is accompanied by a three-fold increase relative to control plants in the capacity for glucose transport by isolated chloroplasts (Häusler et al., 1998). A glucose transporter has previously been characterised in spinach chloroplasts (Schäfer et al., 1977). It catalyzes a facilitated diffusion with highest affinity for D-glucose ($K_m = 20$ mM). It also catalyzed similar rates of D-xylose, D-mannose, and L-arabinose transport and was inhibited by phloretin, a known inhibitor of glucose transport in erythrocytes.

The existence of maltose transporters in the chloroplast envelopes of spinach provides a further potential route for the export of carbon from starch degradation. Maltose is transported into spinach chloroplasts via a mechanism independent of glucose transport, indicating that there are separate maltose and glucose transporters (Herold et al., 1981; Rost et al., 1996). Carbon could also potentially be exported as hexose phosphates. Rates of uptake of hexose phosphates by isolated chloroplasts are usually relatively low (Flügge and Heldt, 1991). However, isolated pea chloroplasts appeared to export hexose phosphates in the dark (Stitt and ap Rees, 1980) and glucose-6-phosphate-dependent starch synthesis was substantially induced in spinach and potato chloroplasts isolated from leaves that had been fed glucose for several days through the petiole (Quick et al., 1995). It remains an open question whether maltose or hexose phosphate transporters play a role in exporting the products of transitory starch degradation.

**E. Conversion of Starch to Sucrose at Night**

The demonstration that carbon can cross the chloroplast envelope either as triose phosphate or as sugars gives rise to the possibility that the products of hydrolytic and phosphorolytic degradation might be exported in different forms and thus have different fates in the cytosol. It is tempting to speculate that hydrolytic degradation gives rise to sugar residues that are exported to support sucrose biosynthesis, whereas phosphorolytic degradation gives rise ultimately to triose phosphate, which is exported from the chloroplast via the TPT to sustain dark respiration (Stitt et al., 1985). Such a division could allow the process of starch degradation to be independently regulated according to the demand for sucrose synthesis and dark respiration. Further, export of free sugar from the chloroplast at night would minimise the requirement for ATP in the stroma to support starch degradation and would be consistent with the apparent absence of hexokinase from chloroplasts. Much further experimental work on the nature and regulation of starch degradation in the chloroplast is clearly required to allow the validity of this hypothesis to be tested. For example, as discussed above, nothing is known about regulatory properties of starch-hydrolysing enzymes that would allow their activities to be modulated to meet a demand for sucrose synthesis. The hypothesis would be invalidated if there is a significant rate of export of hexose phosphate from the chloroplast at night, or if chloroplasts contain significant activity of maltose phosphorylase (Section V.A.4, V.B), since this would allow the products of phosphorolytic degradation to be used in sucrose synthesis.
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VI. Conclusions

Transitory starch is of central importance to many plants, serving as a store of carbohydrate for periods when photosynthesis is not possible. There is a reasonable understanding of the enzymes involved in the synthesis of transitory starch, although it remains unclear exactly how the various isoforms involved combine to give rise to the complex and poorly-understood structure of the starch granule.

The pathway of starch degradation has yet to be firmly established. Many enzymes with potential roles in starch degradation have been identified in leaves, but in most cases there is no convincing evidence that they are involved in transitory starch degradation, or even localised in the chloroplast.

Without a full understanding of the enzymic machinery that mediates starch degradation the regulation of the process can only be guessed at. It is to be hoped that substantial progress can be made on these open questions through the careful analysis of transgenic plants altered in the activities of one or more of the enzymes involved in transitory starch metabolism.

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Chapter 10

Control of Photosynthesis, Allocation and Partitioning by Sugar Regulated Gene Expression

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Summary

A variety of genes, whose products are involved in diverse metabolic pathways and cellular functions, are either induced or repressed depending on the availability of soluble sugars in higher plant cells. This metabolic regulation of gene expression can be viewed at two levels. Firstly, at the cellular level, changes in gene expression result in a modification of anabolic or catabolic processes in response to nutrient availability. This mechanism is important in the control of primary carbon metabolism and nutrient homeostasis within the cell. For example, in mesophyll cells when soluble sugar levels are high there is feedback inhibition on photosynthetic gene expression which can actually override the well documented induction effect of light. At the other extreme, catabolic processes are induced at the level of gene expression when soluble sugar levels are depleted in whole leaves or mesophyll protoplasts. Secondly, when viewed at the whole plant level it becomes apparent that sugar regulation of gene expression plays an important role in source-sink interactions. Productivity in source tissues needs to match demand from sink tissues. Sugars play an integral role in achieving this as they act as signals both for repression of genes coding for photosynthetic enzymes and for induction of genes coding for sink-specific enzymes, such as sucrose synthase, extracellular invertase and granule bound starch synthase.

Since changes in the intracellular levels of carbohydrates result in changes in expression of a variety of genes, then any factor, either local or remote, that affects carbohydrate levels will affect gene expression. Such factors include environmental conditions such as light, temperature, availability of macronutrients and developmental
stage of the plant. In this chapter we will discuss the variety of factors that bring about changes in carbohydrate status in photosynthetic cells and the effect these changes have on gene expression. We will then draw upon the extensive literature in metabolic regulation of gene expression in prokaryotes and lower eukaryotes in order to highlight possible mechanisms that may be involved in the sugar regulated gene expression in plant cells.

I. Introduction

Plant tissues can be divided into those that export (source) and those that import (sink) carbon and nitrogen (Turgeon, 1989). Mature green leaves, seed storage tissue during early post-germinative growth and sprouting tubers are examples of source tissues. Sink tissue can be sub-divided into storage sinks, such as developing seeds and tubers which accumulate various proportions of carbohydrate, proteins and lipids; and metabolic sinks such as meristems and roots which accumulate little or no storage material, but rely on net import of metabolites for growth and development. However, this classification into sink and source organs is not static and depends on genetically fixed developmental processes such as the transition of developing seeds and young leaves from sink to source organs during maturation. It also depends on changes in environmental conditions. The ability of a plant to respond to environmental changes is necessary in order to maximize growth, biomass and reproductive capacity when essential resources such as light, CO$_2$, water and nitrogen are abundant and to compensate when there is a deficit in one or more of these resources. Coordinated development and interaction of source and sink organs is critical in order to achieve this. One of the key processes that needs to be coordinated between source and sink tissue is the photosynthetic production of carbohydrates with their mobilization, allocation and utilization. This acclimation (maintenance of the supply and demand for photosynthetic assimilates in appropriate balance) in response to fluctuating exogenous factors is achieved by short-term modulation of enzyme activities and other proteins along with more long-term changes in expression of genes whose products are involved in the production, mobilization, allocation and utilization of photo-assimilates. These genes are controlled by a variety of factors including developmental, hormonal and environmental signals as well as by carbohydrates, either directly or indirectly, since the products of these genes ultimately modify carbohydrate levels.

II. Carbohydrate Regulation of Gene Expression in Source and Sink Tissues

Photosynthetic gene expression is subject to feedback control by metabolites that signal carbohydrate status of photosynthetic cells. To appreciate this control at the whole plant level it is essential first to consider the factors that regulate carbohydrate status in photosynthetic tissues. Carbohydrate status will be a function of the net production of carbohydrate and the partitioning of assimilated carbon to sink tissues. Therefore, any factor which results in a change in either of these parameters will change the carbohydrate status and so effect a change in photosynthetic gene expression. For example, light intensity, temperature and CO$_2$ availability will directly affect the rate of photosynthesis and so the production of photoassimilates. Availability of essential nutrients such as nitrate or phosphate, will affect the demand for carbon-skeletons for biosynthesis in both source and sink tissues (Paul and Stitt, 1993).

A. Carbohydrate Control of Gene Expression in Source Tissues

A number of model systems have been used to demonstrate that elevated levels of soluble sugars result in repression of photosynthetic gene expression via a feed-back control mechanism. In several cases this control has been demonstrated to occur at the level of transcription. The first example of this type of control came from work using a maize mesophyll protoplast transient expression system in which it was demonstrated that transcription from seven maize

Abbreviations: ADP – adenosine diphosphate; AGP-S – ADP-glucose pyrophosphorylase; AMP – adenosine monophosphate; AMPK – AMP-activated protein kinase; ATP – adenosine triphosphate; CAB – chlorophyl a/b binding protein; cAMP – cyclic adenosine monophosphate; CaMV35S – cauliflower mosaic virus 35S promoter; Cra – catabolite repressor/activator; CRP – cAMP receptor protein; FBP – fructose 1,6-bisphosphate; F1P – fructose 1-phosphate; HXK – hexokinase; PC – plastocyanin; PEP – phosphoenolpyruvate; PTS – phosphotransferase system; RBCS – ribulose bisphosphate carboxylase small subunit; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; SNF1 – sucrose non-fermenting protein kinase; VSP – vegetative storage protein
photosynthetic gene promoters was repressed by sugars or acetate whereas other promoters (e.g. CaMV35S) were not significantly affected (Sheen, 1990). In Arabidopsis, the light induction of the ribulose bisphosphate carboxylase small subunit gene (RBCS) in dark-adapted seedlings is inhibited by growth on 2% sucrose or glucose (Cheng et al., 1992). In rapeseed cell culture 2% sucrose inhibits the light dependent accumulation of the chlorophyll a/b binding protein (CAB) (Harter et al., 1993). Moreover sucrose is also able to repress the early, light independent activation of a plastocyanin gene during early Arabidopsis seedling development (Dijkwel et al., 1996). Sugar repression of photosynthetic genes has also been demonstrated in a variety of other experimental systems including transgenic tobacco expressing invertase in the apoplast resulting in elevated levels of intracellular sugars, a Chenopodium cell culture, and in spinach plants that had been cold-girdled to decrease export of photoassimilates from leaves (von Schaewen et al., 1990; Krapp et al., 1993; Krapp and Stitt, 1995). This work has led to the conclusion that repression of photosynthetic genes by high-carbohydrate-associated metabolic factors represents a basic genetic mechanism (Krapp et al., 1993; Krapp and Stitt, 1995). It should be noted that genes encoding other enzymes including the plastid isoforms of aldolase, triose-phosphate isomerase and phosphoglucomutase, that have a dual role in photosynthesis and respiratory metabolism, are not subject to carbohydrate repression (Krapp and Stitt, 1994) and activities of glycolytic enzymes remain unaltered or increase (Stitt et al., 1991; Krapp and Stitt, 1994).

**B. Influence of Carbohydrate Control in Sink Tissues on Gene Expression in Source Tissues**

Carbon partitioning to sink tissues depends on the plants developmental program which largely dictates the temporal and spatial distribution of storage and metabolic sinks. Superimposed on this are fluctuations in partitioning in response to changing environmental conditions since many forms of environmental stress can restrict availability of essential resources. In general, genes involved in allocation and accumulation of storage reserves show increased expression when carbohydrate levels are high. Expression of these genes decreases when carbohydrate levels decrease.

One of the key mechanisms regulating carbon partitioning to sink tissue appears to be the metabolic regulation of two families of genes encoding enzymes responsible for the initial metabolism of sucrose (Geiger et al., 1996; Koch et al., 1996). Sucrose is the major form of carbohydrate that is transported in many plants. The genes encoding the two enzymes known to be responsible for sucrose cleavage in the phloem and importing tissues of plant cells, namely sucrose synthase and invertase, are themselves regulated by carbohydrate status (Koch et al., 1992; Xu et al., 1996). Both of these enzymes are encoded by a gene family in maize and in both families one class of genes is up-regulated by elevated sugar levels (SUCROSE SYNTHASE1 and INVERTASE2), whereas a second class of genes in the same family is repressed by sugars and upregulated when sugar levels fall (SHRUNKEN1 and INVERTASE1). Interestingly, the two classes of genes from each of these families are also differentially expressed during development. Those genes that are induced by sugars are expressed in sugar-importing tissues and those that are repressed by sugars are induced during reproductive development. Carbohydrate regulation of sucrose synthase and invertase genes from various other species including potato (Salanoubat and Belliard, 1989), rice (Karrer and Rodriguez, 1992), bean (Heim et al., 1993), Chenopodium rubrum (Godt et al., 1995; Roitsch et al., 1995) and Arabidopsis (Martin et al., 1993) have also been reported. The metabolic regulation of sucrose synthase and invertase genes therefore provides a possible mechanism for the regulation of import of carbon into sink tissues. This regulation in itself will effect changes in the carbohydrate status in photosynthetic tissues and in so doing ultimately give rise to changes in photosynthetic gene expression.

Expression of extracellular invertase following wounding or bacterial infection is correlated with repression of photosynthetic gene expression (Sturm and Chripeels, 1990). It has been postulated that the resulting increased hydrolysis of apoplastic sucrose to glucose and fructose may play an important role in coordinating the plants defense response by inducing certain genes encoding for proteins such as proteinase inhibitor II and chalcone synthase, and repressing others such as those involved in photosynthesis (Jang and Sheen, 1994).

The majority of sink genes that respond to sugar related signals are also regulated by a number of
other mechanisms including developmental, stress or environmental signals. This leads to a complex regulatory network in which specific genes are responding to a variety of signals. Further details of metabolic regulation of gene expression in higher plants can be found in recent reviews (Graham, 1996; Koch, 1996; Smeeke and Rook, 1997) and more specifically carbohydrate control of photosynthetic gene expression has also been reviewed (Sheen, 1994; Stitt et al., 1995).

III. Influence of Other Metabolites on Sugar Regulated Genes

Photosynthetic acclimation is suggested to be caused by accumulation of carbohydrates in the source leaves resulting in feedback inhibition of photosynthesis (Azcon-Bieto, 1983; Blechschmidt-Schneider et al., 1989). Therefore, any developmental or environmental factor which results in increased levels of carbohydrate in source tissues could cause a repression of photosynthesis.

Photosynthetic acclimation has been observed in some instances when plants are grown at elevated CO₂ (Stitt et al., 1991; Bowes, 1994; Van Oosten and Besford, 1996). Acclimation of tomato plants to elevated levels of CO₂ in which an initial increase in photosynthesis is followed by a long term decrease has recently been linked to sugar repression of photosynthesis (Van Oosten and Besford, 1994). Exposure of tomato plants to either elevated CO₂ or sucrose resulted in decreased levels of nuclear encoded mRNA for photosynthetic enzymes, whereas repression of chloroplast genes was less pronounced (Van Oosten et al., 1994; Van Oosten and Besford, 1996). The similar effects of elevated CO₂ and sugars has led to the proposal that the initial increase in photosynthesis after exposure to elevated CO₂ results in accumulation of sugars (if sink strength is insufficient) and this, in turn, results in repression of nuclear encoded photosynthetic genes (Van Oosten and Besford, 1994, 1996).

Deficiency of nitrogen or phosphorous results in accumulation of carbohydrates in mature leaves and roots within hours after withdrawal of these nutrients (Thorsteinnson and Tillberg, 1990; Henry and Raper, 1991). This implies that nitrogen or phosphorous deficiency limits utilization of carbohydrate more than it limits photosynthesis. In nitrogen-deficient shoots and roots of tobacco seedlings, levels of hexose phosphates and 3-phosphoglyceric acid are elevated and upon feeding of exogenous sucrose, protein, Rubisco and chlorophyll content is decreased in shoots as nitrogen is recycled (Paul and Stitt, 1993).

Transcripts encoding enzymes involved in nitrate assimilation and starch synthesis increase in response to elevated levels of soluble sugars in photosynthetic tissues. Sucrose can substitute for light in eliciting an increase in steady state levels of nitrate reductase mRNA in dark-adapted green Arabidopsis plants (Cheng et al., 1992). In the same experiment, sucrose was shown to override the light induction of the RBCS gene. Both light and exogenous sucrose repress accumulation of asparagine synthetase and increase nitrate reductase mRNA levels in Arabidopsis (Lam et al., 1994). The partial elimination of sucrose repression of asparagine synthetase expression by amino acids (Lam et al., 1994) suggests that nitrogen:carbon ratio rather than carbon alone is responsible for the metabolic regulation of at least this carbohydrate regulated gene. Control of gene expression by metabolites may therefore regulate major fluxes of carbon and nitrogen in plants.

Allosteric regulation of the starch biosynthetic enzyme ADP-glucose pyrophosphorylase by phosphate and 3-phosphoglyceric acid is a well established mechanism for regulation of starch biosynthesis. An additional level of control appears to act at the level of gene expression. Genes encoding ADP-glucose pyrophosphorylase (AGP-S) (Müller-Röber et al., 1990) and granule bound starch synthase (Visser et al., 1991) have been shown to be induced by high levels of sugars in sink tissues. A similar mechanism appears to operate in source tissues. Feeding glucose to autotrophic cell cultures of Chenopodium or cold-girdling of spinach leaves results in a rapid increase in the AGP-S transcript (Krapp et al., 1993; Krapp and Stitt, 1995). However, other factors must also be involved in the large stimulation of starch accumulation after adding glucose or inhibiting export since ADP-glucose pyrophosphorylase activity does not increase significantly (Krapp and Stitt, 1994). The decrease in activity of the plastidic (but not the cytosolic) isofrom of starch phosphorylase when glucose is added to Chenopodium cells suggests that carbohydrates may also regulate the breakdown of starch (Krapp and Stitt, 1994).

Transgenic tobacco plants with very low nitrate reductase activity have been used to provide evidence that nitrate acts as a signal to induce organic acid
metabolism and repress starch metabolism in tobacco (Scheible et al., 1997). Addition of nitrate to these plants results in an increase in transcripts for phosphoenolpyruvate carboxylase, cytosolic pyruvate kinase, citrate synthase, and NADP-isocitrate dehydrogenase even though the nitrate is not being reduced and entering metabolism proper. The same treatment results in a decrease of the ADP-glucose pyrophosphorylase transcript and enzyme activity, and starch decreases in the leaves and roots. This work has led to the proposal that nitrate acts as a signal to initiate coordinated changes in carbon and nitrogen metabolism.

IV. Hormones and Sugar Regulation

Animal systems provide the best examples of how sugars and hormones can interact to regulate a given process. One such example is the glucose-induced insulin secretion from pancreatic β-cells (for review, see Holz and Habener, 1992). To trigger secretion of insulin, glucose has to be phosphorylated by glucokinase and processed by glycolysis and the Krebs cycle. The consequent increase in ATP relative to ADP is thought to mediate a glucose induced depolarization of the β-cell membrane possibly by inhibiting the activity of metabolically regulated potassium channels resulting in an influx of calcium into the cell. The increase in intracellular calcium activates the exocytosis of vesicular insulin. This regulation of insulin secretion by glucose availability is modified by other processes including the inhibitory and stimulatory action of a large number of hormones and neurotransmitters thus allowing a high plasticity of the response reaction (Holz and Habener, 1992).

Do similar interactions between sugars and hormones exist in plants as have been demonstrated in animal systems? Plant hormones have been shown to have an effect on carbohydrate metabolism in both source and sink tissue. For example the activities of cytosolic sucrose phosphate synthase and fructose-1,6-bisphosphatase, which determine to a great extent the amount of sucrose in a leaf, are affected by exogenous application of hormones. Gibberellin application to soybean leaves increases sucrose phosphate synthase activity while the application of abscisic acid inhibits fructose-1,6-bisphosphatase activity (Brenner and Cheikh, 1995).

A recent report of work carried out on a Chenopodium rubrum cell suspension culture has shown that cytokinin treatment results in induction of a cell wall invertase gene (CIN1) and a hexose transporter gene at the mRNA level (Ehness and Roitsch, 1997). The increase of cell wall invertase transcripts corresponds to higher steady state protein level and enzymatic activity of an invertase. Cytokinins did not affect the expression of two putative intracellular invertases (CIN2 and CIN3), sucrose synthase or two other hexose transporters present in the cell suspension culture showing the specificity of this response (Ehness and Roitsch, 1997). The increased transcription of CIN1 and the hexose transporter results in higher rates of sugar uptake in induced compared to non-induced cells. CIN1 mRNA is also induced by cytokinin in tissues of C. rubrum plants thus indicating a physiological role for this hormone in the control of carbohydrate partitioning in plants (Ehness and Roitsch, 1997). Since cell wall invertase is also induced by glucose and 6-deoxyglucose in the same C. rubrum cell suspension culture (Roitsch et al., 1995) it would appear that sugars and hormones are involved in the induction of this enzyme at the level of gene expression. In contrast to the induction effect of cytokinin, physiological concentrations of ethylene were found to reduce the enzyme activity of cell wall invertase by 25–47% and this was paralleled by repression of the corresponding gene (Linden et al., 1996).

Dual control by sugars and hormones has also been reported for rice α-amylase and the soybean vegetative storage protein (VSP) gene. The rice α-amylase gene is repressed by sugars and abscisic acid and can be induced by gibberelin (Karrer and Rodriguez, 1992), whereas the activation of VSP gene expression by sugars and methyljasmonate is inhibited by auxins (DeWald et al., 1994).

The possibility that sugar sensing and ethylene signal transduction pathways are integrated was recently highlighted by a report from Sheen’s laboratory on the characterization of Arabidopsis gin (glucose insensitive) mutants (Zhou et al., 1998). Arabidopsis gin mutants were isolated due to their ability to germinate and grow on 333 mM glucose. It was revealed that Arabidopsis mutants that produce significantly increased levels of ethylene (eto: ethylene overproduction) or mutants which show an ethylene over-production phenotype due to a defect in the ethylene signal transduction pathway (ctr: constitutive triple response) behave like gin mutants on 333 mM glucose. Furthermore AVG (L-α-(2-
aminoethoxyvinyl-glycine), an inhibitor of ethylene biosynthesis, reverts the gin phenotype to wild type in the eto mutants and in at least one of the gin mutants (gin1.1). However, CAB1 gene expression, which is insensitive to glucose in the gin1.1 mutant, is sensitive to glucose in the ethylene mutants as it is in wild type. This work therefore suggests that ethylene may be playing an important role in the response of germinating seedlings to high levels of glucose whereas it does not appear to be involved in the carbohydrate repression of CAB gene expression.

V. Sugar Sensing

All organisms regulate the flux of carbon through key metabolic pathways in order to balance carbohydrate metabolism with nutrient uptake, cellular osmotic potential, growth and physiology. In microorganisms, sugar sensing enables cellular metabolism to be adjusted in response to changes in carbon source availability in the external environment. Glucose is the preferred carbon source for the majority of microorganisms. The availability of glucose acts as a specific signal to repress genes encoding proteins involved in the utilization of alternative carbon sources. Our understanding of sugar sensing and sugar signal transduction pathways is much more advanced in bacteria and yeast than it is in plants or animals.

A. Distinct Sugar Sensing Mechanisms Exist in Evolutionary Divergent Prokaryotes

Carbon catabolite repression is a universal phenomenon in prokaryotes. Multiple mechanisms of control exist within a single cell and distinct mechanisms exist between evolutionary divergent species. The classic picture of catabolite repression in prokaryotes is that involving a cAMP-dependent mechanism. In E. coli this involves a phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS) that serves multiple functions one of which is sugar sensing. This system transports, phosphorylates and detects a variety of sugars. In so doing, it initiates sugar metabolism and regulates a number of physiologically important processes including the phenomena of inducer exclusion, inducer expulsion and catabolite repression (Saier et al., 1995). The PTS consists of several phosphoryl transfer proteins (Enzyme I, Hpr, IIA, IIB, and IIC) that must be sequentially phosphorylated before sugar substrates can be transported and concomitantly phosphorylated. Enzyme I and Hpr are general (non-sugar-specific), soluble, cytoplasmic components of the system while the IIA, IIB and IIC components are sugar-specific membrane bound bifunctional permeases/kinases. Adenylyl cyclase is believed to be allosterically activated by the phosphorylated form of the glucose loaded IIA protein (IIA<sup>Δk-P</sup>). When active, it synthesizes cAMP, which binds to the cAMP receptor protein (CRP). The cAMP-CRP complex binds directly to DNA to activate transcription. Catabolite repression results when glucose in the medium causes a dephosphorylation of II<sup>A<sub>Δk-P</sub></sup>, thereby deactivating adenylyl cyclase. This in turn, causes the cytoplasmic cAMP concentration to diminish which causes the cAMP-CRP complex to dissociate from the DNA, thus resulting in catabolite repression (Saier et al., 1995; Saier, 1996).

In recent years, cAMP-independent catabolite repression mechanisms have been delineated in bacteria (Saier et al., 1995). Recent studies have shown that E. coli and Salmonella typhimurium also utilize other mechanism which are completely independent of the cAMP-CRP complex to control transcriptional initiation of genes encoding the key enzymes of the major pathways for carbon metabolism (Ramseier, 1996). These pathways include the Embden-Meyerhof and Entner-Dudoroff glycolytic pathways (subject to catabolite activation) and the Krebs cycle, glyoxylate shunt and gluconeogenic pathways (subject to catabolite repression). The pleiotropic transcriptional regulatory protein that controls expression of these central metabolic genes has been termed the ‘Catabolite repressor/activator’ (Cra). In vitro Cra-binding experiments have shown that micromolar concentrations of fructose 1-phosphate (F1P) or millimolar concentrations of fructose 1,6 bisphosphate (FBP), but not other catabolites or gluconeogenic intermediates, displaced the Cra protein from the promoters of all Cra regulated genes examined (Ramseier, 1996). Cra exerts a negative effect on glycolytic genes and a positive effect on genes encoding Krebs cycle, glyoxylate shunt and gluconeogenic enzymes. Under glucose-rich conditions, the concentrations of FBP and F1P increase and these interact with the Cra protein and act to dissociate it from target genes thus mediating catabolite activation of glycolytic genes and catabolite repression of Krebs cycle, glyoxylate shunt and gluconeogenic genes. In regulating such a variety of
genes, the Cra protein effectively controls the direction of carbon flux through metabolic pathways in enteric bacteria.

In evolutionary distinct species, still other mechanisms have evolved to control carbon catabolite repression. For example, in Bacillus subtilis and other Gram-positive bacteria cAMP is absent and instead, intracellular carbon metabolites such as fructose-1,6-bisphosphate (FBP) and gluconate-6-phosphate, the common intermediates of the Embden-Meyerhof and Entner-Duodoroff pathways, respectively, promote catabolite repression. The mechanism of repression involves a metabolite activated, ATP-dependent protein kinase that phosphorylates a serine residue in HPr, the small phosphocarrier protein of the PTS. HPr-(ser-P) then binds to various protein targets (transcription factors, carbohydrate permeases and hydrolytic enzymes) to regulate their activities.

It is evident, then, that several different catabolite repression and inducer control mechanisms have evolved in prokaryotes, many which are independent of cAMP. Some of these mechanisms may be operative in eukaryotic microorganisms, higher plants and animals. The variety of cAMP independent mechanisms for catabolite repression, particularly those which involve universal metabolite intermediates as effector molecules, provide models for similar mechanisms in higher plants. If this is the case then the rapid accumulation of sequence data for model plant species such as Arabidopsis and rice along with already complete genome sequences for some bacteria (e.g., Bacillus subtilis, Haemophilus influenzae and Mycoplasma genitalium) should allow identification of putative regulatory components from plants in the near future. The task will then be to prove functional significance of these genes and proteins in plants.

**B. Sugar Sensing in the Yeast Saccharomyces cerevisiae**

As in E. coli, carbon catabolite repression in S. cerevisiae has been found to involve a number of distinct regulatory mechanisms. Addition of fermentable sugars to S. cerevisiae grown on non-fermentable carbon sources causes a variety of short-term and long-term regulatory effects. The regulatory switch is characterized by a rapid transient activation of cAMP synthesis, mobilization of the storage carbohydrate trehalose and induction of genes encoding glycolytic enzymes and ribosomal proteins. Furthermore, high-affinity sugar transport and key gluconeogenic enzymes are rapidly inactivated and transcription of a variety of genes encoding respiratory, gluconeogenic and other enzymes is repressed (Gancedo, 1992; Trumbly, 1992). Several signaling pathways are involved in mediating the above phenomena of which the classic glucose repression pathway and the Ras-cAMP pathways are the best studied. Sensing of the available sugar by both these signaling pathways requires transport and phosphorylation but no further metabolism. S. cerevisiae contains two homologous hexokinases, PI (Hxk1) and PII (Hxk2), that can phosphorylate both glucose and fructose, and a single glucokinase (Glk1), only capable of phosphorylating glucose (Lobo and Maitra, 1977). Activation of the Ras-cAMP pathway by glucose or fructose is dependent on sugar phosphorylation by any of these three hexose kinases. In contrast, glucose-dependent transcriptional repression exerted through the main glucose repression pathway is primarily dependent on the hexokinase PII. Deletion of the hexokinase PII gene abolishes glucose repression, whereas deletions of the hexokinase PI or glucokinase genes have no effect. Overexpression of hexokinase PI, but not glucokinase, relieves the requirement for PII in establishing glucose repression suggesting a function of the hexokinases in triggering catabolite repression that is separate from enzyme activity (De Winde et al., 1996, and references therein).

A recent report has demonstrated that the main glucose repression pathway involves two distinct mechanisms: an initial rapid response that can be mediated through any one of the three S. cerevisiae hexose kinases; and a second, long-term repression response that specifically requires hexokinase PII for glucose repression and either hexokinase PI or hexokinase PII for fructose repression (De Winde et al., 1996). Both the hexokinase PI and the glucokinase genes are themselves repressed by glucose and fructose. However, the repression of the hexokinase PI gene by fructose is transient which is in agreement with the preference of PI for fructose as substrate and its role in long-term fructose repression. These results demonstrate that sugar sensing and establishment of catabolite repression in S. cerevisiae is controlled by an interregulatory network, involving all three yeast hexose kinases and the Ras-cAMP pathway (De Winde et al., 1996).

As discussed below, it would now appear that at least part of the hexokinase sensing mechanism may
have been conserved and adapted for sugar sensing in higher plants.

Characterization of the phenotypes of additional *S. cerevisiae* mutants have resulted in the identification of two signaling pathways which operate downstream of sugar sensing. Although one of these pathways is responsible for repression and the other for de-repression of genes that are subject to carbon catabolite control there is substantial crosstalk between the two. Two protein complexes have been identified in the repression pathway. One of these (REG2/REG1/GLC7 type 1 protein phosphatase) mediates a signal by regulating a protein phosphatase activity but no substrate for this activity has as yet been identified. The second complex (SSN6/TUP1) functions as a general repressor of transcription through modulation of chromosome structure. Interaction of the SSN6/TUP1-complex with DNA is mediated by the MIG1 DNA binding protein, which somehow directs binding of the complex to specific sites in the promoter region of genes that are repressed by glucose.

Derepression of gene expression in the absence of glucose is necessary to allow alternative carbon sources to be utilized. This derepression process involves another regulatory pathway containing at least two protein complexes. The first complex (SNF1/SNF4) mediates a signal through activity of the SNF1 protein kinase of which there are plant and mammalian homologues (see below). The second complex (SWI/SNF1) consists of at least ten proteins which modulate chromatin conformation and affect expression of a variety of genes. Targeting of this complex to specific gene promoters is mediated by transcription factors. It is not yet known how the SWI/SNF complex receives information from the SNF1 complex in the derepression pathway. The SNF1 complex also directly affects the repression pathway by inactivating the MIG1 repressor protein. Similarly the GLC7 protein phosphatase complex from the repression pathway inhibits the activity of the SNF1 kinase complex.

Our understanding of the mechanisms outlined above, although detailed relative to our knowledge in plants, is still far from complete. One important gap still remaining to be filled is how the metabolite signals involved are sensed and transduced into the regulatory pathway. As discussed above, hexokinase has been implicated as the sugar sensor triggering the repression pathway but a mechanism of action still has to be defined.

An alternative mechanism involving AMP:ATP ratios for signaling intracellular nutritional status to the de-repression pathway has recently emerged through biochemical studies of the *S. cerevisiae* SNF1 kinase (Wilson et al., 1996). The mammalian AMP-activated protein kinase (AMPK) is a homologue of SNF1 kinase. AMPK is made up of three subunits and all of these have homologues that are part of the SNF1 complex. AMPK is activated by the elevation of the cellular AMP:ATP ratio, which occurs during cellular stress in mammalian cells. The mechanism of activation involves allosteric regulation by AMP, promotion of phosphorylation by an upstream protein kinase (AMPK kinase) and inhibition of dephosphorylation of AMPK. The proposed mode of action in mammalian cells is that AMPK functions as a ‘fuel gauge’ (Hardie and Carling, 1997). When AMPK detects a low fuel situation it protects the cell by switching off ATP-consuming pathways (e.g. fatty acid synthesis and sterol synthesis) and switching on alternative pathways for ATP generation (e.g. fatty acid oxidation). SNF1 is rapidly activated by phosphorylation on removal of glucose from *S. cerevisiae*, and can be inactivated by protein phosphatases and reactivated by mammalian AMPK kinase, thus demonstrating that the mammalian AMPK and *S. cerevisiae* SNF1 protein kinase cascades are highly conserved (Wilson et al., 1996). The only apparent difference in these two complexes is that the SNF1 complex is not regulated allosterically by AMP in vitro. However, multiple levels of control by AMP:ATP ratio exist for the mammalian complex and a similar situation may exist for the yeast complex. Based on this work, Wilson et al. (1996) put forward a new model for the role of SNF1 protein kinase in carbon catabolite repression. This model proposes that adenine nucleotides, rather than an interaction of glucose with hexokinase, are the metabolic signals which indicate the lack of glucose in the medium. These signals trigger activation of SNF1 kinase and de-repression of glucose repressed genes.

AMPK/SNF1 homologues also exist in plants but their function has not yet been defined. SNF1 homologues from rye and tobacco can complement the *snf1* mutation in *S. cerevisiae* (Alderson et al., 1991; Muranaka et al., 1994). Unlike the plant homologue, the mammalian AMPK does not complement *snf1* regulatory mutants (Woods et al., 1994). Additional SNF1 homologues have been cloned from barley, wheat and *Arabidopsis* (Halford
et al., 1992; Le Guen et al., 1992; Sano and Yousseflian, 1994). A tobacco SNF1 homologue, NPK5, constitutively activates expression of the glucose repressible SUC2 gene in S. cerevisiae snf1 mutants (Muranaka et al., 1994). However, expression of NPK5 does not rescue mutants disrupted in the SNF4 gene, the product of which associates with SNF1 and is necessary for maximal activity of the kinase in yeast. These results indicate that NPK5 can substitute for SNF1 in the SNF1/SNF4 complex in S. cerevisiae snf1 mutants (Muranaka et al., 1994). Carbohydrate starvation of wheat seedlings results in expression of the SNF1 homologue WPK4 (Sano and Yousseflian, 1994). Light and cytokinins also induce WPK4 gene expression which suggests that this particular kinase may be involved in more than one signal transduction pathway.

An AMPK specific peptide based kinase assay (Davies et al., 1989) has been used to identify AMPK-like activities in extracts from avocado, potato, carrot, pea, oilseed rape, wheat and cauliflower (MacKintosh et al., 1992). Immunological studies indicate that the cauliflower kinase is the homologue of the rye and tobacco SNF1-like protein kinases (Ball et al., 1995). Like the S. cerevisiae SNF1, the cauliflower kinase is not sensitive to AMP. In all other respects, including being able to inactivate mammalian acetyl-CoA carboxylase and HMG-CoA reductase, it is similar to the mammalian AMPK. The physiological substrate for the plant AMPK/SNF1 homologue is not yet known but it has been shown that the cauliflower kinase inactivates bacterially synthesized HMG-CoA reductase from Arabidopsis by phosphorylating the protein at the same site that is phosphorylated in the mammalian HMG-CoA reductase (Dale et al., 1995).

Much work still has to be done to establish the in vivo role(s) of the plant SNF1 homologues. It is possible that this family of protein kinases plays a role in signal transduction of metabolic status in plant cells. Alternatively, or as well, these kinases could play an important role in the post-translational control of key metabolic enzymes as is the case in mammalian cells. Two such enzymes in plant cells are sucrose phosphate synthase which catalyzes a key step in sucrose biosynthesis in source tissues and nitrate reductase which catalyzes the first step in nitrogen assimilation. In spinach leaves both these enzymes are regulated by phosphorylation by multiple protein kinases (Chapter 8, Foyer et al.; McMichael et al., 1995). One of three nitrate reductase kinases detected in extracts from spinach leaves has identical properties to other SNF1-like enzymes (Douglas et al., 1997). It is, therefore, likely that plant SNF1 homologues do play an important role in regulating metabolic processes in source tissue through post-translational control of key metabolic enzymes.

Experiments involving antisense and over-expression of plant SNF1 homologues are currently underway in several laboratories and these along with specific kinase assays and monitoring expression of appropriate genes under different metabolic conditions should provide useful information on the role of the plant kinase. In addition, identification of plant homologues to other components in the SNF1 signal transduction pathway, such as SNF4 or a possible upstream kinase, along with their functional analysis would provide additional evidence for a conserved mechanism of carbon catabolite repression between plants and yeast.

C. Sugar Sensing in Plants—the Case for Hexokinase

Studies on the sugar mediated repression of the glyoxylate cycle and photosynthetic genes has led to the suggestion that sugar sensing in plants involves a hexokinase (Graham et al., 1994a,b; Jang and Sheen, 1994). In cucumber cell cultures, the malate synthase and isocitrate lyase genes are repressed in the presence of glucose, fructose or sucrose and de-repressed when intracellular levels of these sugars fall below a critical threshold (Graham et al., 1994a,b). A cucumber mesophyll protoplast transient expression system was used to demonstrate that these same genes are de-repressed at the transcriptional level upon carbohydrate depletion (Graham et al., 1994a). The glucose analogues 2-deoxyglucose and mannose, which are phosphorylated by hexokinase but not further metabolized by the glycolytic pathway, cause repression even though intracellular amounts of sucrose, glucose and fructose fall below the critical levels normally associated with de-repression (Graham et al., 1994b). In contrast to glucose, fructose or sucrose, 3-O-methylglucose, an analogue of glucose that is taken up by the cells but is not phosphorylated by hexokinase, does not result in repression of malate synthase and isocitrate lyase. These results implicate hexokinase or events associated with the hexokinase reaction in the initial sugar sensing step. However it should be noted that experiments with hexose analogues alone do not rule out the possibility of a stereospecific hexose receptor
operating independently of hexokinase.

Using a *C. rubrum* cell culture, Krapp et al. (1993) demonstrated that 6-deoxyglucose or 3-O-methylglucose (both imported into the cell but not phosphorylated by hexokinase) had no effect on *RBCS* gene expression, whereas glucose at the same concentration caused severe repression. It was concluded from this work that transport of carbohydrates does not provide the signal for regulation of *RBCS* and that phosphorylation of glucose is necessary. Using a maize mesophyll protoplast transient expression system, Jang and Sheen (1994) demonstrated that 2-deoxyglucose causes repression of photosynthetic gene promoters whereas 3-O-methylglucose has no effect on expression. The importance of hexokinase in sugar sensing was further demonstrated by feeding experiments using mannheptulose, an apparently specific inhibitor of hexokinase, which was shown to block the repression effect of glucose and 2-deoxyglucose (Jang and Sheen, 1994). However, more recent work using a *C. rubrum* cell culture has reported that both D-glucose and 6-deoxyglucose treatments result in a similar decrease in *RBCS* mRNA levels and an increase in the mRNAs for a sink specific cell wall invertase and for phenylalanine ammonia-lyase, a key enzyme of defense response (Ehness et al., 1997). An explanation for these conflicting reports is not apparent.

Cloning of two *Arabidopsis* genes, *AtHXK1* and *AtHXK2* has allowed overexpression and antisense experiments to be carried out in order to establish the in vivo involvement of hexokinase in the response of seedlings to elevated levels of glucose (Jang et al., 1997; Jang and Sheen, 1997). Wild type seedling growth is inhibited in the presence of 333 mM glucose whereas antisense seedlings with reduced levels of hexokinase show less inhibition of growth. Photosynthetic genes such as *RBCS* and *CAB* are also more strongly repressed by glucose in wild type than in antisense plants. Overexpression of the hexokinase in transgenic seedlings results in increased sensitivity to 333 mM glucose relative to wild type. The authors’ interpretation of these results together with other results using the yeast hexokinase, is that hexokinase is a sugar sensor. Decreasing hexokinase protein decreases the capacity of seedlings to sense glucose hence they grow better on 333 mM glucose and do not exhibit the same degree of glucose repression of photosynthetic gene expression as is observed in wild type. Increased levels of hexokinase lead to increased sugar sensing capacity and increased sensitivity to 333 mM glucose. Another interpretation is that 333 mM glucose is toxic to seedlings, possibly because it results in a massive flux of hexose sugar into the hexose phosphate pool. This might lead to a sequestration of phosphate which could disturb the balance of cellular metabolism. A third possibility is that high concentrations of hexose phosphates in the cell have deleterious effects. Decreasing hexokinase activity will decrease this flux whereas increasing hexokinase activity will increase the flux. The crucial experiment which supports the view that hexokinase is acting as a sugar sensor involved overexpressing the *S. cerevisiae HXK2* gene (which is implicated in sugar sensing in *S. cerevisiae*) in *Arabidopsis*. If the yeast hexokinase were to exhibit sugar sensing in *Arabidopsis* then transgenic seedlings should be more sensitive than wild type to 333 mM glucose. In fact, the opposite phenotype was observed in which the transgenic seedlings were less sensitive to 333 mM glucose than wild type even though the total hexose phosphorylation activity was significantly greater than that in wild type seedlings. The interpretation given for this is that the yeast hexokinase competes effectively for the same substrate as the endogenous glucose sensing hexokinases resulting in reduced sensitivity to glucose.

The hexokinase under- and over-expressing plants were also used to investigate the role of hexokinase in the regulation of sugar inducible nitrate reductase. *Arabidopsis* lines over-expressing *AtHXK* show higher steady state nitrate reductase mRNA levels in response to glucose than wild type seedlings, thus suggesting that hexokinase is also important in signaling expression of sugar-inducible genes. However, other effects such as increased flux of hexose sugars into glycolysis cannot be ruled out.

D. Sugar Transport and Signaling

The generation of a signal by metabolism of a sugar is one way in which carbohydrate status is perceived. An alternative or additional mechanism could involve intra- or extracellular receptors. In the simplest case, a DNA binding protein might act as a sensor and transmit the signal directly to the respective gene as in the case of some hormone induction in mammalian cells. Alternatively, the receptor could be part of a sugar transport system (possibly similar to the PTS system in *E. coli*) in which increased or decreased levels of sugars lead to a chain of signaling events
which ultimately cause a sugar specific response.

The yeast \textit{SNF3} and \textit{RGT2} genes encode putative glucose transporters with high similarities to mammalian, yeast and plant glucose transporters, but differ from them in having a long C-terminal extension. This extension may be located in the cytoplasm and could serve as a signaling domain (Celenza et al., 1988; Marshall-Carlson et al., 1990; Bisson et al., 1993). Mutational analysis has shown that both \textit{SNF3} and \textit{RGT2} may act as sugar sensors and are involved in the induction of glucose induced \textit{HXT} gene regulation in yeast (Ozcan et al., 1996).

Furthermore this analysis has shown that \textit{SNF3} and \textit{RGT2} might act as a pair of glucose receptors recognizing either high (\textit{RGT2}) or low (\textit{SNF3}) levels of extracellular glucose. The authors suggest that as in the case of the bacterial PTS system the generation of the sugar signal is coupled to the transport of this sugar and that the components of the sugar signaling pathway are present in the cell even in the absence of the sugar. In this model, the activity of the glucose sensor becomes the limiting factor of the signaling system (Ozcan et al., 1996).

Despite the cloning of a number of sugar transporter genes from plants (Sauer and Tanner, 1993) a sugar transporter/sensor protein equivalent to the yeast \textit{SNF3} or \textit{RGT2} has not yet been identified in plants.

Experiments using non-metabolizable sugar analogues provide evidence for at least two different signal perception mechanisms in plants. As discussed above, source-related genes, e.g. \textit{RBCS} and \textit{CAB}, can be repressed by 2-deoxyglucose, or mannose but not by 3-O-methylglucose (Jang and Sheen, 1994). In contrast, the more sink metabolism-related sucrose synthase, invertase and class I patatin genes are induced by 3-O-methylglucose, 6-deoxyglucose or 2-deoxyglucose (Godt et al., 1995; Roitsch et al., 1995; Ehness et al., 1997; Martin et al., 1997). In addition, the induction of a sugar regulated chimeric patatin class I/\textit{uidA} (B33GUS) gene in transgenic \textit{Arabidopsis} plants by the glucose analogs 6-deoxyglucose and 3-O-methylglucose shows that at least some sugar signaling pathways in an intact plant system do not require sugar phosphorylation (Martin et al., 1997).

VI. Signal Transduction

Specific components of the signal transduction pathways involved in regulation of plant genes by sugars have not yet been identified. As discussed above (Section V.B.) one family of protein kinases that may play a role in the transduction of sugar signals are the plant homologues of the AMPK/\textit{SNF1} protein kinase which have been shown to be essential for post-translational control of key
biosynthetic enzymes in mammalian cells and de-repression of genes that are subject to carbon catabolite repression in yeast (Hardie and Carling, 1997).

As in other systems, phosphatases, kinases, calcium and calmodulin appear to be involved in the transduction of sugar signals in plants (Lue and Lee, 1994; Takeda et al., 1994; Ohto et al., 1995).

VII. Sugar Response Elements in Gene Promoters

In contrast to the detailed characterization of various photosynthetic gene promoters in relation to their response to light, there have been few reports characterizing their response to carbohydrates. Sheen (1990) used the maize protoplast transient expression system to demonstrate that sucrose repression of a number of maize photosynthetic genes was mediated by positively acting promoter elements upstream of the TATA box, but no specific consensus sequences were defined. More recently (Urwin and Jenkins, 1997), transient expression experiments with protoplasts from primary leaves of Phaseolus vulgaris have been used to identify promoter sequences involved in sucrose repression of the P. vulgaris RBCS2 gene. A region of this promoter from position –203 to –187 contains sequences resembling elements involved in the sugar stimulation of transcription of several plant and mammalian genes. These include the sucrose response elements (SURE elements) of the patatin gene promoter (Grierson et al., 1994). The SURE elements are similar to elements defined in the sporamin and β-amylase genes of sweet potato both of which are induced by sucrose (Ishiguro and Nakamura, 1992). The –203 to –187 region of the RBCS2 gene implicated in sugar repression also has remarkable similarity to carbohydrate response elements (ChoRE) found in the promoters of mammalian genes induced by sugars, one of which is the L-type pyruvate kinase gene. Whether the RBCS2 –203 to –187 promoter region is sufficient to mediate a sucrose response, or whether any functional similarity exists with the elements described above, requires further research.

VIII. Sugar Sensing Mutants

A number of laboratories have designed various genetic screens in the model plant Arabidopsis thaliana in an effort to isolate mutants in the signal transduction mechanisms involved in sugar mediated repression and induction of gene expression. In the majority of cases young seedlings have been screened as these respond to exogenous sugars in various ways and it is also possible to look at large mutagenized populations. One such screen has exploited the fact that intracellular sugar concentrations increase dramatically in Arabidopsis seedlings grown on high concentrations of sucrose and growth limiting amounts of nitrate compared to when seedlings are grown on high concentrations of sucrose and sufficient amounts of nitrate. The increase in intracellular sugars results in an increase in anthocyanins and a decrease in chlorophyll. Using high sucrose, low nitrate conditions a number of carbohydrate insensitive (cai) mutants have been isolated which do not show the same decrease in chlorophyll and increase in anthocyanins as in the wild type but do show the same increase in intracellular sugar levels (S. Boxall, T. Martin and I. A. Graham, unpublished). As mentioned previously (Section V.C), growth of Arabidopsis seedlings on 333 mM glucose results in developmental arrest and this is overcome in plants that have been antisensed for hexokinase I or hexokinase II (Jang et al., 1997). These same conditions have been used to isolate glucose insensitive (gin) mutants which are less sensitive to 333 mM glucose in the growth medium (Zhou et al., 1998).

The Arabidopsis plastocyanin (PC) gene is transiently activated in both the light and dark during early seedling growth and this expression can be repressed by sucrose (Dijkwel et al., 1996). A transgene screening approach using a PC gene promoter-luciferase reporter gene construct has been used to isolate Arabidopsis mutants that are disrupted in the sugar repression of the plastocyanin PC gene during early seedling growth (Dijkwel et al., 1997). Sucrose uncoupled (sun) mutants show no or reduced repression of the transgene and, importantly, the endogenous PC, CAB and RBCS mRNA levels are similarly insensitive to sucrose repression. Studies of the affect of 2 mM 2-deoxyglucose on the mature rosette stage Arabidopsis have shown that whole plant photosynthesis, plastocyanin gene expression and total extractable Rubisco activity is unaffected in one mutant sun6 (Van Oosten et al. 1997). This treatment results in reduced photosynthesis, PC gene expression and Rubisco activity in wildtype
Arabidopsis.

Many photosynthetic genes that are subject to sucrose repression are also subject to light regulation via phytochrome. The presence of sucrose in the growth medium results in altered PHYA responses in the sun6 mutant compared to wildtype. This suggests that the mechanisms of sugar signaling and light signaling are interrelated (Dijkwel et al., 1997).

Arabidopsis mutants disrupted in the induction of specific genes by sugars have also been isolated. For example, the β-amylase gene is induced by sugars in Arabidopsis and low level beta amylase (lba) mutants have been isolated which do not show this induction response (Mita et al., 1997a,b). In another example the patatin class I promoter, which is induced by sugars in potato, was transferred to Arabidopsis where it was found to be similarly regulated. Transgenic Arabidopsis harboring a patatin promoter-β-glucuronidase transgene were used to isolate mutants showing a reduced sucrose response (rsr) (Martin et al., 1997). The demonstration that the promoter of the potato tuber storage protein is regulated by sugars in the same manner in Arabidopsis demonstrates a high degree of conservation between the mechanisms underlying sugar signaling in plants. Characterization of Arabidopsis mutants will allow us to identify the key regulatory components involved in sugar signaling. One application of this knowledge will be manipulation of sugar signaling and resource allocation in crop species in order to increase yields of current plant products and to over-ride regulatory mechanisms that may inhibit high yields of genetically engineered novel plant products.

IX. Conclusions

In this chapter we have summarized our current understanding of carbohydrate regulation of gene expression and how this relates at the cellular and whole plant level to control of photosynthetic gene expression. Plants contain several sugar signaling cascades, one of which is important in source tissues, possibly involving hexokinase, and another which is independent of hexokinase possibly located at the plasma membrane in sink tissues. The components of a hexokinase independent sugar signaling pathway could be as in yeast a sugar sensing transporter protein (Oezcan et al., 1997) or simply a sugar sensing protein as the first component of a signaling cascade. The analysis of Arabidopsis mutants disrupted in sugar induced responses should help in understanding how plants sense their sugar status and coordinate sink and source metabolism.

We have given many examples of how metabolites regulate gene expression in other organisms in an attempt to emphasize the variety of mechanism that may operate in plants. It is reasonable to predict that at least some of these mechanism will be conserved across phyla and indeed a precedent for this has already been set in the case of the regulation of heat shock genes (Spena and Schell, 1987). In higher plants various mechanisms appear to have either evolved or been conserved and adapted throughout evolution to meet the physiological requirements of source and sink tissues. Future efforts to manipulate production in source tissues, and ultimately plant yield, will require a thorough understanding of the regulatory mechanisms involved.

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Chapter 11

Intercellular Transport and Phloem Loading of Sucrose, Oligosaccharides and Amino Acids

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Summary

Photoassimilate export from the mesophyll and the processes underlying phloem loading are central to the efficient growth, competitive ability, and reproductive success of a plant. The assimilate flux out of the leaf is regulated on a number of levels. For example, structural aspects including the spatial organization of individual cell types within the leaf and the extent of the symplasmic connections between these cell types control these fluxes at the cellular level. Phloem loading can follow a symplasmic route, or involve an apoplastic step within the vicinity of the companion cell-sieve element (CC-SE) complex. In the latter case, apoplastic transfer is regulated by the capacity of the individual cell types to engage in efflux or uptake (retrieval) of photoassimilates.

Within the autotrophic tissues of the leaf, photoassimilate flow may be regulated through feedback mechanisms that can modify biochemical pathways, plasmodesmal conductivity, and membrane transport properties. At the membrane transport level, molecular techniques have led to the isolation and characterization of transporters operating at the site of phloem loading. These studies allowed for the molecular manipulation of such transport systems and now offer a powerful method to advance our understanding of the events that underlie both phloem loading and photoassimilate allocation. The recent discovery that plasmodesmata can mediate the cell-to-cell transport of macromolecules suggests that these unique structures may play a role not only in assimilate transport, but also in the integration of cellular processes. A model is proposed where regulatory macromolecules move cell to cell, within the leaf, as well as long-distance, via the phloem, to serve in the coordination and regulation of physiological events taking place in source and sink tissues.

I. Introduction

Assimilates synthesized in the mesophyll cells of mature leaves are the fuel for metabolism and provide the building blocks for growth in all non-photosynthetic plant tissues, including developing leaves, stems, roots, flowers and seeds. Within the leaf, photosynthate is translocated into the conducting tissue of the phloem and then distributed throughout the plant. This process—fundamental for functioning of the specialized, composite higher plant organism—has attracted the attention of plant scientists for decades, sparked passionate discussions on the mechanisms and pathways involved, and is now beginning to be explored by more detailed molecular investigation. Progress in our understanding of the underlying mechanisms associated with assimilate transport and allocation is currently due in large part to the molecular cloning of membrane transporters which has permitted the detailed molecular characterization of their involvement in phloem loading. In addition, new insights into the functioning of plasmodesmata have expanded our understanding of how these intercellular channels, which are unique to plants, may control symplasmic transport and intercellular communication in higher plants. The available evidence suggests that both membrane transport and symplasmic communication and/or flux of assimilates are essential components for photosynthate transfer from the mesophyll into the vascular tissue where subsequent phloem loading takes place.

In this chapter we describe recent progress in our understanding of photoassimilate transport through the use of molecular and cell biological approaches. These findings are discussed in relation to the complex process of assimilate transfer from the site of photosynthesis within the mesophyll to the site of loading into the companion cell-sieve element (CC-SE) complex of the minor vein network in the leaf.

II. Photoassimilate Movement from the Mesophyll to the Phloem

As illustrated in Fig. 1, the basic morphology and anatomy of the leaf are optimized for efficient light harvesting and \( \text{CO}_2 \) fixation in the mesophyll tissue. Photoassimilates—sugars and amino acids—produced in individual cells have to pass through one, or several, cell layers (Fig. 1B,C) to arrive at a minor vein which functions as the site of assimilate loading into the long-distance transport conduit (Fig. 1D)—the sieve tube system of the phloem (Geiger, 1975). In essence, two pathways are available for the transfer of assimilates: the apoplasm and the symplasm. Assimilates may exit across the mesophyll plasma membrane into the apoplastic space (cell walls and regions exterior to the plasma membrane) from where they can move, by the process of diffusion, into a neighboring minor vein (Fig. 2A,B). Alternatively, as mesophyll cells are interconnected by plasmodesmata (complex cytoplasmic bridges that provide for a spatial continuum of the cytoplasmic domain between cells to form the symplasmic space), assimilates can also diffuse within the symplasm from cell to cell. Following an evaluation of the experimental evidence for the participation of these two routes, we consider the implications of these two pathways in terms of likely regulatory mechanisms.

**Abbreviations:** BS – bundle sheath; CC-SE – companion cell-sieve element complex; EM–electron microscopy; PCMBS–p-chloromercuri-benzenesulfonic acid; SEL–size exclusion limit; SER – sieve element reticulum; TMV-MP – tobacco mosaic virus movement protein; VP – vascular parenchyma
Fig. 1. Assimilate export in higher plants. A, Schematic representation of the plant, illustrating the general relationship between autotrophic leaves and heterotrophic tissues. Photoassimilates produced in the mesophyll are loaded into the minor veins (circle) and the phloem sap then moves into the major vein (dart) of the autotrophic leaf (source) for long-distance translocation (arrows) to the heterotrophic shoot and root (sink) tissues. (Not to scale) B, Illustration of the differences in the minor vein network present in leaves of C₃ (Coronilla varia) and C₄ (Euphorbia maculata) plants. Note the presence of the predominate bundle sheath cells lining the minor veins in the C₄ leaf. C, Cross sectional drawing of a typical C₃ leaf, illustrating the spatial relationship between the abaxial palisade mesophyll (parenchyma) and adaxial spongy mesophyll cells and the minor vein. The bundle sheath cells (shaded) form a special interface between the mesophyll and the vascular tissues of the xylem and phloem. Shaded arrows depict the pathway followed by photoassimilates from the mesophyll to the site of loading in the phloem. D, Ultrastructural details associated with the companion cell-sieve element complex at the site of loading within a minor vein of a tobacco (Nicotiana tabacum) leaf (× 5,200).
A. Photoassimilate Flux through the Apoplastic Pathway

Exchange of solutes across membranes is a general feature of all living cells. Therefore, it is not surprising that a number of studies have demonstrated that leaf discs and mesophyll protoplasts release sugars and amino acids into the surrounding medium—a process which presumably occurs via carrier-mediated mechanisms (Fig. 2) (Secor and Schrader, 1984; Daie, 1989; Laloi et al., 1993; Perez-Alfocea and Larher, 1995). However, in the intact leaf the situation appears to be more complex. It is unfortunate that, because of inherent experimental limitations, only a limited number of attempts have been made to quantify assimilate pools in the apoplasm of source leaves (Ntsika and Delrot, 1986). For this reason, it is not clear how much of the assimilates that are released into the apoplasm, across the mesophyll plasma membrane, move within this compartment for direct transfer to the minor vein. In this context it would be important to ascertain whether there are differential rates of release from mesophyll cells adjacent to the minor veins versus those more distantly located (see Fig. 1C).

The basic question that remains to be resolved is whether the release of sugars and amino acids is controlled by unique efflux mechanisms, or occurs by leakage through carriers normally engaged in transport into the symplasmic compartment (i.e., retrieval of solutes from the apoplasmic compartment). Certainly, uptake into the mesophyll has been demonstrated in numerous systems (Maynard and Lucas, 1982; Wilson and Lucas, 1988; Theodoropoulos and Roubelakis-Angelakis, 1991; Flora and Madore, 1993; Flora and Madore, 1996; Van Bel et al., 1996). Furthermore, both sugar and amino acid carriers have been studied using plasma membrane vesicles isolated from leaves (Bush, 1989, 1993; Gallet et al., 1989; Tubbe and Buckhout, 1992; Buckhout, 1994). Finally, promoters from mono- and disaccharide transporters have been shown to drive expression in mesophyll tissue (Sauer et al., 1994) and the mesophyll plasma membrane has similarly been shown to contain carrier protein for sucrose (Lemoine et al., 1989). These findings are consistent with the concept that carriers regulate the level of sugars within the mesophyll apoplasm. This situation most probably applies also for assimilates such as amino acids, as well as oligosaccharides and polyols in plant species where these compounds are synthesized.

A careful analysis of the sucrose flux in the Ricinus cotyledon established that, in this system, at least 50% of the sucrose mobilized in the endosperm followed an entirely apoplastic pathway through the mesophyll into the minor veins (Orlich and Komor, 1992). However, whereas the importance of an apoplastic route through the mesophyll has been firmly established in the Ricinus cotyledon, extrapolating from this experimental system to the situation of a transpiring leaf may be difficult.

It has often been proposed that the flow of assimilate to the veins would have to be separated from the rapid transpirational flow of water within the leaf (Stitt, 1996). This problem could be avoided if the water coming up the xylem were to enter the vapor phase in the immediate vicinity of the xylem vessels (Boyer, 1985) or be channeled through specific symplasmic routes to evaporation sites next to the stomatal pores. In addition, major transpirational water flow away from the xylem may occur through the walls of the bundle sheath extension cells (see Fig. 6A), followed by lateral transport along the epidermis (Canny, 1993). As minor veins generally lack these extensions, diffusion of solutes through the mesophyll apoplasm may not be disturbed by transpirational water flow (Canny, 1990). Finally, as the apoplastic pathway for assimilate flow could provide a ready source of carbon and nitrogen for invading microbial pathogens, plants would have to react through the development of a variety of defense strategies (Bent, 1996; Dangl et al., 1996; Jackson and Taylor, 1996).

Additional evidence for the presence of an apoplastic sucrose pool within the leaf has been provided by studies on transgenic plants that express and target a yeast acid invertase into the cell wall. In such tobacco (Von Schaewen et al., 1990), tomato (Dickinson et al., 1991) and potato plants (Heineke et al., 1992), starch and sugar levels were found to increase in the leaves, export was reduced and source leaves underwent early senescence. The physiological changes are consistent with cleavage of sucrose, bound for export, in the apoplasm followed by retrieval of monosaccharides predominantly into mesophyll cells. Interestingly, a severe phenotype was not observed in Arabidopsis plants expressing an extracellular invertase (Von Schaewen et al., 1990). Finally, the development of secondary plasmodesmata
Pathways for Photoassimilate Export

A

Group I - symplasmic continuity

B

Group II - near symplasmic isolation of CC-SE complex

C

Group II - apoplastic diffusion barrier at the bundle sheath

Fig. 2. Potential apoplastic and symplasmic pathways for photoassimilate flux from the mesophyll into the companion cell-sieve element (CC-SE) complex. S denotes a transported photoassimilate; open arrows represent diffusion through the symplasm, via plasmodesmata; closed arrows illustrate diffusion through the apoplasm, via hydrated cell walls. Membrane-bound active and passive sugar transport systems are represented by solid and open circles, respectively. Suberin deposition in the radial cell walls of the bundle sheath is depicted by dark shading in C; these deposits appear to form a barrier to diffusion, within the apoplasm, between the cells of the vascular tissue and the mesophyll.
was found to be inhibited in tobacco leaves which overexpress cell wall invertase (Ding et al., 1993). This latter observation strongly suggests that, at some level, plasmodesmata formation is regulated and coupled to assimilate export.

As the yeast invertase was expressed in these transgenic plants under the control of the 35S CaMV promoter, which is known to drive transcription in mesophyll and phloem tissues, and as the explicit cellular location of the acid invertase within these tissues was not established, it is not clear which apoplastic sucrose pool was intercepted in these transgenic plants that were expressing yeast acid invertase. The range of observed phenotypes may be explained by a varying degree of transgene invertase activity in the minor vein apoplasm, near the presumptive site of loading from the apoplastic compartment into the phloem. Much more would be learned about the relative importance to export of sucrose pools in the apoplasm of the mesophyll and the phloem, by overexpressing the cell wall invertase in the respective tissue via promoters specific for the mesophyll and the phloem (e.g., as was done recently with a viral movement protein in potato: Olesinski et al., 1996).

In some species, transfer of assimilates from the mesophyll to the minor vein via the apoplasm may be blocked by suberin deposition in the cell walls of the bundle sheath (Canny, 1990; Robinson-Beers and Evert, 1991; Botha, 1992; Evert et al., 1996). In this situation, solutes in the mesophyll apoplasm would not be able to pass directly to the site of loading in the minor vein, but rather, they would have to be retrieved by plasma membrane carriers and then pass, symplasmically, across the bundle sheath boundary (Fig. 2C).

It is unfortunate that for the intact photosynthetic leaf it has not been feasible to directly quantify the amount of assimilates that are released into the mesophyll apoplasm. Hence, the importance of the apoplastic route for assimilate transfer across the mesophyll for delivery into the minor vein remains to be resolved. The identification of sucrose and amino acid permeases that may be involved in the controlled transfer of photoassimilates into the apoplasm would certainly open the way for more definitive experiments.

B. Photoassimilate Flux through the Symplasmic Pathway

Mesophyll cells are generally highly interconnected by plasmodesmata establishing a semi-selective symplasmic pathway which usually extends from the mesophyll through the bundle sheath and into cells of the minor vein. In some plant families, this high degree of symplasmic continuity extends all the way up to the long-distance transport pathway, which comprises files of individual companion cell-sieve element complexes (CC-SE; see Fig. 1D, Fig. 2A). (For a detailed description of plasmodesmal structure, the reader is referred to Lucas et al., 1993a; Overall and Blackman, 1996; Ding, 1997).

Dye injection studies have shown that, in general, plasmodesmata are open for the passage of small molecules (< 1 kDa) and will permit diffusion of such molecules (metabolites) from the mesophyll into the minor vein (Robards and Lucas, 1990). Thus, it is now widely accepted that assimilates may travel from the mesophyll into the minor vein through this symplasmic route. Nevertheless, it has to be borne in mind that the presence of plasmodesmata, as revealed by electron microscopy (EM) studies, does not necessarily indicate that an intercellular pathway is available for the passage of solutes. It is well known that plasmodesmata can be closed by callose deposition (Robards and Lucas, 1990); e.g., after wounding or pathogen attack. Even more important for the regulation of symplasmic photoassimilate transfer is the finding that plasmodesmal conductivity (open or closed state) may be regulated by pressure gradients between neighboring cells (Oparka and Prior, 1992).

The importance of plasmodesmata to the control of assimilate export may be reflected in the coordination of secondary plasmodesmal formation in leaves and the onset of assimilate export. For example, the transition from importing sink tissues to exporting source tissues was found to coincide with the formation of secondary plasmodesmata in the mesophyll of maize (Evert et al., 1996) and with modifications of plasmodesmata in the minor veins of cucurbit (Volk et al., 1996). These observations indicate that formation and/or modification of plasmodesmata can affect the capacity of the symplasmic pathway for the transfer of assimilates. Thus, a dynamic control over plasmodesmal properties could exert a powerful structural level of control over photosynthate export. Indeed, plasmodesmal frequency has been widely used as a measure of symplasmic continuity and taken as an indication of the capacity for symplasmic solute exchange between cells (Van Bel et al., 1988; Beebe and Evert, 1992; Van Bel et al., 1992).
This simplified view of plasmodesmal frequency should be perceived with some caution (Robards and Lucas, 1990; Van Bel and Oparka, 1995). At present there is a paucity of experiments that address the operational characteristics of plasmodesmata. For example, we do not have available experimental values for the conductivity of individual mesophyll plasmodesmata in terms of the cell-to-cell diffusion of assimilates. In the absence of this type of information it is presently impossible to develop a quantitative model for the symplasmic route from the mesophyll into the minor veins. In this respect—and this also applies to apoplastic fluxes across the mesophyll—our knowledge has remained more metaphysical than biophysical in nature.

As symplasmic transfer, through plasmodesmata, of small molecules such as photoassimilates is assumed to be driven by diffusion, downhill concentration gradients from the mesophyll to the minor vein should exist for the respective metabolite. For the exchange of C\textsubscript{4} intermediates between the mesophyll and the Kranz cells (specialized bundle sheath cells; see Fig. 3A), such diffusion gradients have been established (Weiner et al., 1988). Unfortunately, the situation is less clear for photoassimilates which are actually exported from the leaves both in C\textsubscript{3} and C\textsubscript{4} plants, due to a lack of information on the relevant assimilate concentrations in the cytoplasm. As the cytoplasmic compartment in the mesophyll is below 10%, and most of the cell volume is occupied by vacuoles, exact data on cytoplasmic sucrose concentrations are difficult to obtain, although feasible for a uniform population of culture cells (Preißer et al., 1992). But it would be difficult, if not impossible, to extrapolate from cell suspension experiments to conditions in the cytoplasm of mesophyll, bundle sheath and phloem parenchyma cells within intact source leaf tissues.

An approach designed to gain empirical insight into the process of symplasmic assimilate transfer used transgenic tobacco plants expressing the viral movement protein of tobacco mosaic virus (TMV-MP). In this experimental system, the TMV-MP has the capacity to increase the size exclusion limit (SEL) of mesophyll plasmodesmata (Wolf et al., 1991; Wolf et al., 1989). If diffusion through plasmodesmata were rate-limiting for sucrose export, this TMV-MP-induced increase in SEL would be expected to result in a stimulation of translocation from the source leaves. However, analysis of carbohydrate levels in the mesophyll and determination of [\textsuperscript{14}C]-export rates revealed that sugar and starch levels were elevated and export was reduced in these TMV-MP expressing plants (Lucas et al., 1993b; Olesinski et al., 1995). The impact of these results on the concepts of how photosynthate export may be regulated will be discussed later.

These findings on transgenic tobacco plants and other recent insights into plasmodesmal structure and function have challenged the viewpoint that plasmodesmata are simple pores for diffusion of small molecules (Lucas et al., 1993a; Eipel, 1994). Microinjection studies have now established that viral movement proteins (Fujjwara et al., 1993; Noueiry et al., 1994; Waigmann et al., 1994) and the plant transcription factor, KNOTTED 1 (Lucas et al., 1995), are able to traffic through plasmodesmata and mediate the cell-to-cell transfer of nucleic acids. These results stimulated the development of novel hypotheses regarding the supracellular nature of plants (Lucas et al., 1993a) and the control of plant development and physiology (Lucas, 1995; Mezitt and Lucas, 1996).

C. Disruption to Symplasmic Transfer of Photoassimilates in the Maize Mutant \textit{sxd1}

In C\textsubscript{4} plants such as maize, CO\textsubscript{2} is fixed in the mesophyll into C\textsubscript{4} acids (malate) by phosphoenolpyruvate carboxylase (Fig. 1B, Fig. 3A). Malate passes through plasmodesmata into the bundle sheath cells where CO\textsubscript{2} is released for subsequent fixation in the chloroplasts. Interestingly, although the chloroplasts in both bundle sheath and mesophyll cells have the capacity to store starch, the enzymes involved in sucrose synthesis appear to be predominantly located in the mesophyll (Lunn and Furbank, 1997). Thus, plasmodesmata play a central role in facilitating the exchange of intermediates involved in CO\textsubscript{2} fixation and sucrose synthesis. (For more details on various aspects of C\textsubscript{4} photosynthesis the reader is referred to chapters 19 (Leegood), 20 (Dengler and Taylor), 21 (Sage and Pearcy), 22 (Monson and Rawsthorne) and 23 (Cushman et al.).)

The importance of plasmodesmata and the symplasmic route in mediating the delivery of sucrose to the site of loading in the phloem gained strong support from a recent study by Russin et al. (1996). These workers reported that sucrose levels in a mutant maize line, termed \textit{sxd1}, were elevated whereas phloem export was dramatically reduced. An ultrastructural examination of wild-type and \textit{sxd1} leaf tissues revealed that, in this mutant line, the plasmodesmata interconnecting the
bundle sheath and vascular parenchyma cells had undergone some form of blockage (Fig. 3B, C).

Careful inspection of the transmission electron micrographs of these plasmodesmata revealed that they appeared to be sealed by apposition of additional wall material (Fig. 3C). A similar phenomenon has been reported in studies conducted on guard cells (Wille and Lucas, 1982), where sealing of plasmodesmata connecting guard cells to the neighboring epidermal cells appears to be essential for the onset of stomatal function. In sxd1 mutant plants, plasmodesmal closure at the bundle sheath–vascular boundary was restricted to the leaf blade; normal plasmodesmal structures within the leaf base...
apparently permitted the delivery of sufficient photosynthate to apical tissues to alleviate the severity of the sxd1 mutation on plant development.

Two possible scenarios for the manner in which the sxd1 mutation might elicit the observed phenotype are presented in Fig. 3A. As the sealing of the plasmodesmata occurs later in leaf development (W. Russin, personal communication) and is restricted to plasmodesmata interconnecting bundle sheath–vascular parenchyma (or companion) cells (BS-VP/CC) in the leaf blade (Russin et al., 1996), the sxd1 mutation may be located within a repressor gene which, in the mutant state, allows ectopic expression of a unique developmental program in the bundle sheath cells. This program then orchestrates the directed apposition of new wall material (WAP in Fig. 3A) across the orifice of plasmodesmata located at the BS-VP/CC boundary. The finding that plasmodesmata interconnecting bundle sheath cells, as well as between bundle sheath and mesophyll cells, were structurally unaffected by the sxd1 mutation implicates the involvement of positional-dependent information (Fig. 3A; left schematic).

Once the plasmodesmata connecting BS-VP/CC cells have been sealed, symplasmic passage of sucrose into the VP/CC would be prevented, thereby causing the observed increase in soluble sugars in sxd1 maize plants. In response to elevated sugar levels, chloroplasts in both BS and mesophyll cells would deposit enhanced quantities of starch, as was observed by Russin et al. (1996).

Another interesting feature of the sxd1 mutant was the finding that during tissue preparation for EM, VP/CC cells were prone to plasmolysis. This would be consistent with closure of the main route for sucrose entry to the site of phloem loading, which would result in a severe depletion of sugar levels within phloem cells. Under these circumstances, the high osmotic potential within these cells would allow plasmolysis to take place in a highly cell-specific manner.

An alternate explanation for the structural and physiological changes induced by sxd1 is that this mutation alters a critical aspect of sucrose loading into the sieve element. This impediment to sucrose loading could reflect the synthesis of a dysfunctional transporter that is engaged in either the release of sucrose into the apoplasm or its energy-dependent loading into the SE. In this situation, sucrose levels within the VP/CC and BS cells would be expected to quickly increase to a level that may activate a sugar-induced regulatory cascade. A pleiotropic effect of this cascade could be the activation of the genes involved in sealing the plasmodesmata at the BS-VP/CC boundary (Fig 3A; right schematic).

Identification of the SXD1 gene will provide a test for the two scenarios depicted in Fig. 3A. In addition, knowledge of the cellular functions controlled by SXD1 may well provide a powerful tool for the further analysis of the impact of the symplasmic pathway on physiological and developmental processes in plants.

D. Oligosaccharides and Polyols as Transport Sugars

Most of the experimental data mentioned above were obtained from studies conducted on plants that are either important agricultural plants, such as maize, potato and tomato (Stitt, 1996), or Ricinus which lends itself to the study of phloem transport (Komor et al., 1996). All these plants share sucrose as the dominant sugar in the phloem translocation stream. Indeed, a survey of 97 monocot and dicot plant families indicated that sucrose is the exclusive form of sugar translocated in the phloem of some 50 plant families, whereas in a further 33 families other forms of polysaccharides are translocated (Zimmermann and Ziegler, 1975). Raffinose-type oligosaccharides and polyols (e.g., mannitol and sorbitol) represent a major transport compound in 14 and 6 plant families, respectively (Table 1). In every case, sucrose is also present in the translocation stream and at times it is present at a high concentration.

With the presence of various sugars in the leaf, the question arises as to which sugars are synthesized in the mesophyll for transport to the minor veins and which are subsequently loaded into the sieve tube. For example, in Cucumis melo leaves sucrose and galactinol are found in both the mesophyll and the minor vein in high concentration, but raffinose and stachyose are mostly restricted to the cells of the minor vein (Haritatos et al., 1996). These findings are consistent with sucrose and galactinol moving from the mesophyll into the phloem where they act as precursors for the synthesis of raffinose and stachyose in the intermediary cells (see Fig. 4) (Beebe and Turgeon, 1991). Similarly, in olive plants, sucrose, galactinol and mannitol are found in the mesophyll, but only stachyose and sucrose are exported (Flora and Madore, 1993). However, in species, sucrose and mannitol are rapidly labeled...
Table 1. Major sugars and polyols translocated in the phloem of plants. The number of plant families is listed where the respective assimilate was found as a dominant (10–30%) or an additional (<10%) component in the phloem sap. Data were compiled from Zimmermann and Ziegler (1975), van Die and Tamme (1975), Fukumotra and Chino (1982) and Weiner et al. (1991). Values in parentheses represent the number of families where the respective assimilate is the exclusive transport form.

<table>
<thead>
<tr>
<th>Phloem sap constituents</th>
<th>Dominant sugar</th>
<th>Minor constituent</th>
<th>Constituent not detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>83 (50)</td>
<td>14</td>
<td>–</td>
</tr>
<tr>
<td>Raffinose-type sugars</td>
<td>14 (0)</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>Polyols</td>
<td>6 (0)</td>
<td>33</td>
<td>58</td>
</tr>
</tbody>
</table>

Fig. 4. Models for phloem loading. A, Direct loading of sucrose (S) by the sucrose-H+ transporter (SUT1) into the sieve element (SE) after release of sucrose from phloem parenchyma or bundle sheath cells (PP/BS), as suggested by immunolocalization of SUT1 to the SE plasma membrane in Solanaceae species. B, Localization of the sucrose-H+ carrier (SUC2) from Arabidopsis and Plantago in the companion cell (CC) would indicate loading of sucrose via the CC plasma membrane and symplasmic flow of sucrose from the companion cell into the sieve element through branched plasmodesmata. Movement of macromolecules (MM) from the companion cell into the sieve element (SE) occurs in both cases. C, Hypothesis for symplasmic phloem loading via a novel polymer trap mechanism (see text for details). (Modified after Grusak et al., 1996.)
in $[^{14}\text{CO}_2]$ pulse-chase experiments and then both forms appear to move from the mesophyll to the minor vein for export from the leaf (Flora and Madore, 1996).

Little information is available on the mechanisms which mediate uptake and/or retrieval of oligosaccharides and polyols across the plasma membrane in plant species which synthesize raffinose-type sugars and polyols. That carriers for polyols exist was shown by uptake studies using leaf discs from parsley (Flora and Madore, 1996) and plasma membrane vesicles from celery petioles (Salmon et al., 1995). Raffinose-induced membrane depolarization also indicate the presence of uptake mechanisms for this type of oligosaccharide in mesophyll cells of Catharanthus and Ocimum (Van Bel et al., 1996). The challenge ahead will be to develop appropriate molecular cloning techniques to further advance our knowledge of the nature and evolution of these oligosaccharide transport systems.

E. Amino Acid Transfer from the Mesophyll to the Phloem

Depending on plant species and nitrate availability, net amino acid synthesis may proceed to a considerable extent in mesophyll tissues (Andrews, 1986). This allows for direct use of photosynthetic energy in the reduction process and favors shoot over root growth (Burns, 1994; Beck, 1996). As discussed above for sucrose, amino acids may use apoplastic and/or symplasmic routes for transfer into the minor veins. The respective route taken may depend on the nitrogen supply which can result in large variations in the levels of individual amino acids within the leaf (Shelp, 1987; Schobert and Komor, 1989).

Several classes of amino acid transporter genes have been cloned by yeast complementation studies using cDNAs from Arabidopsis (Frommer et al., 1993; Hsu et al., 1993; Kwart et al., 1993; Fischer et al., 1995; Frommer et al., 1995; Rentsch et al., 1996; Chen and Bush, 1997). Using this information and sequence similarities to known amino acid transporters, these plant systems can be classified into two major superfamilies: the plant cationic amino acid transporters (CATs) and the amino acid transporter family 1 (AFT1). The CAT genes are related to the mammalian family of cationic amino acid transporters. To date, only a single member of this family has been identified from plants. The gene encoding this transporter, AtCAT1, was cloned by complementation of a yeast histidine transport mutant (Frommer et al., 1995). Sequence analysis indicated that AtCAT1 is likely composed of 14 putative membrane spanning domains. Based on indirect evidence, it would appear that AtCAT1 functions as a high affinity secondary active transport system for basic amino acids; however, the system also appears to recognize a broad spectrum of other amino acids, albeit with a lower affinity (Frommer et al., 1995).

Members of the AFT1 superfamily, which represents the largest and best characterized plant amino acid transporter family, were also cloned by complementation of yeast mutants. Detailed sequence analyses predict that these transporters contain 10 membrane-spanning domains (Rentsch et al., 1998). Several members of the AFT1 family have now been shown to function as amino acid $\text{-H}^+$ symporters (Boorer et al., 1996; Boorer and Fischer, 1997). Using analysis of substrate specificity, in combination with sequence similarities, four amino acid transporter subfamilies have been identified: namely the proline transporter (ProT), the amino acid permease (AAP), the lysine-histidine transporter (LHT), and the auxin-resistant clones (AUX1). As their name implies, the two members of the ProT subfamily, identified from studies on Arabidopsis, exhibit a high transport affinity for proline (Rentsch et al., 1996). In contrast, the members of the AAP subfamily recognize a wide range of different amino acids, including amides and ureides (Fischer et al., 1995; Boorer et al., 1996; Boorer and Fischer, 1997). The AtLHT1 subfamily was identified by homology searches in the database and was subsequently shown to transport lysine and histidine with high affinity (Chen and Bush, 1997). The AtAUX1 subfamily was discovered from studies performed on an auxin-resistant mutant of Arabidopsis (Bennett et al., 1996). The current hypothesis is that, because auxins are structurally very similar to tryptophan, during plant evolution an AAP amino acid transporter homolog may have been mutated to yield an auxin transporter (AUX1). Analyses of CAT and AFT1 expression patterns indicate that individual family members are expressed within specific tissues (Kwart et al., 1993; Fischer et al., 1995; Rentsch et al., 1996). Organ-dependent differences in specificity of amino acid uptake mechanisms were also observed in Ricinus (Schobert et al., 1997). However, the expression of a given amino acid transporter can be limited to a particular cell type, or it can overlap with other transporter
genes. Naturally, expression patterns deduced from Northern blot analysis provide only a limited amount of information, and must be followed up by careful in-situ and immunolocalization studies. Studies of this nature will be required to determine which of these transport systems are involved in the transfer of amino acids from the mesophyll to the phloem translocation stream.

III. Phloem Loading

Before reviewing the progress made with respect to identification of the molecular components which drive assimilate accumulation in the CC-SE complex, a definition of the term phloem loading needs to be clearly stated, as it pertains to the present discussion. In the past two decades, the Münch (1930) pressure flow hypothesis has been generally recognized as the only model which can explain all of the experimental observations regarding assimilate flow in the phloem of higher plants. For the sake of clarity, we will use the term phloem loading (Geiger, 1975) to describe the processes and underlying mechanisms: (i) involved in the uptake of assimilates into the CC-SE complex, and (ii) required for the generation and maintenance of the thermodynamic gradient necessary to drive the mass flow of assimilates out of the source leaf.

A. Cellular Organization of the Phloem Long-Distance Transport Pathway

The phloem in the vascular bundles of angiosperms is comprised of parenchyma cells and the companion cell-sieve element (CC-SE) complex, which functions as the long-distance transport pathway (Fig. 1). The CC and SE differentiate after division of a precursor cell, but remain intimately connected by specialized plasmodesmata (Fig. 5B). The SE undergoes a pattern of differentiation resulting in features unique among living plant cells (Sjölund, 1997). The nucleus, vacuole and Golgi apparatus of the differentiating SE become completely degraded and structurally simplified plasmodesmata yield plasma membrane-lined pores in walls that interconnect contiguous SEs (sieve plates and lateral sieve areas), thereby forming the functional sieve tube system. The plasma membrane, a parietally displaced endoplasmic reticulum termed the sieve element reticulum (SER), modified plastids and mitochondria remain within the mature, functional SE (Evert, 1990).

Unlike the situation in animals where a muscle-driven pump generates flow through the vessels which represent a space outside of living cells, long-distance transport in the phloem of higher plants occurs within the highly specialized living SEs. There, as in the animal system, fluid flow is driven by a differential in hydrostatic (turgor) pressure. Turgor within the SEs is generated by the accumulation (loading) of photoassimilates, which results in a lowering of the osmotic potential within the SE. The subsequent reduction in the SE water potential establishes a gradient with respect to the surrounding tissues, which causes water to enter the SE from the nearby xylem conducting elements (Fig. 1). As the CC-SE complex is located deep within the cellular architecture of the vein, this osmotically induced inflow of water does not cause an expansion in SE volume, but rather, it generates a significant increase in turgor pressure. The removal of specific organelles and the anchoring of the remaining organelles to the plasma membrane thus allows efficient turgor-driven mass flow, through the low-resistance sieve plate pores, from sites of assimilate production, termed sources, to sites of assimilate utilization, termed sinks (Fig. 1A).

The differences in SE turgor between the sites of phloem loading and unloading result in mass transport of phloem sap in the direction of this turgor gradient; recorded flow rates range from 0.3 to 3 m/h (Köckenberger et al., 1997). These rates are quite remarkable given the small diameter of the SE (average of 10–20 μm) (Sjölund, 1997). Viewed from another perspective, if we assume an average length of 200 μm for an individual SE, then its entire content would be displaced (in the direction of mass flow) up to 5 times every second!

Considering that the SEs of higher plants are enucleate, their maintenance as living cells over a long period of time appears to rely on the adjacent CCs which are characterized by a dense cytoplasm and an abundance of mitochondria and ribosomes (Fig. 5A, B). The metabolites and proteins needed to sustain sieve tube function are thought to be synthesized in the CC prior to being transported into the sieve tube through specialized plasmodesmata that interconnect these two cell types (Fig. 5A, B). Analysis of sieve tube sap has shown that it contains more than 100 polypeptides (Fisher et al., 1992; Nakamura et al., 1993; Sakuth et al., 1993). Lectins (Bostwick et al., 1992), redox regulatory proteins (Ishiwatari et al., 1995; Szederkenyi et al., 1997), chaperones (Schobert et al., 1995), protein kinases...
(Nakamura et al., 1995), protease inhibitors (Murray and Christeller, 1995) and amylase (Wang et al., 1995) have been shown to be constituents of SEs, either by immunolocalization or by their presence in isolated sieve tube sap.

Recent experiments have provided experimental evidence for the hypothesis that phloem proteins have the capacity to mediate their own cell-to-cell transport through plasmodesmata. Squash phloem proteins were isolated from sieve tube sap and then size-fractionated; fractions ranged from 10 to above 100 kDa. After microinjection into squash mesophyll cells these proteins moved from cell to cell and potentiated the movement of fluorescently labeled 20 kDa dextran (Balachandran et al., 1997). Direct microinjection of high-molecular-weight fluorochromes into the SEs of the fascicular stem phloem of *Vicia* confirmed that the plasmodesmata connecting the CC-SE complex are open for the passage of molecules having an SEL greater than 10 kDa but less than 40 kDa (Kempers and Van Bel, 1997). These findings indicate that: (i) significant import of proteins occurs into the sieve tube system, and (ii) unfolding may be required for proteins larger than 20 kDa to pass the plasmodesmata connecting the CC to the SE. For this purpose, agents such as molecular chaperones may well be essential to mediate refolding of these proteins following their transport into the sieve tube membrane (Schobert et al., 1995; Balachandran et al., 1997). Chaperones may also be required for targeting and integration of integral membrane proteins into the sieve tube membrane.
B. Modes of Phloem Loading

The number of plasmodesmata connecting the CC-SE complex to the surrounding cells within the minor vein may vary widely across diverse plant families. These structural differences in the minor vein configuration have been used to classify higher plants into two major groups, with implications on the mechanism of phloem loading. These groups have been defined as follows (Gamalei, 1988):

Group I: The CC-SE complex is highly connected with phloem parenchyma cells, via plasmodesmata, and thus, a symplasmic continuity is maintained between the mesophyll and the CC-SE complex (Fig. 2A). It has been noted that many members in this group transport additional oligosaccharides in the phloem, together with, or in greater amounts than, sucrose (Gamalei, 1991). The inference has been drawn that phloem loading in group I plants is primarily via a symplasmic mechanism.

Group II: A limited number of plasmodesmata connect the CC-SE complex with the surrounding cells of the minor vein (Fig. 2B, C) which results in a constriction of the symplasmic connection between photoassimilate-producing cells and the CC-SE. Here, the inference has been drawn that in group II plants, phloem loading occurs via an apoplasmic step between the phloem parenchyma and CC-SE complex.

An elegant hypothesis has been advanced to explain, mechanistically, how symplasmic phloem loading may operate (Turgeon and Gowan, 1990). This process has been referred to as ‘polymer trapping’ and is thought to involve a combination of metabolic and plasmodesmata specializations at the bundle sheath/CC-SE boundaries. For plants such as cucurbits, it has been proposed that plasmodesmata connecting the bundle sheath to the intermediary cell (a specialized form of the companion cell) do not allow the passage of metabolites above 600 Da. However, the plasmodesmata connecting the intermediary cell to the SE are thought to be structurally and/or functionally modified to allow diffusion of larger molecules. Figure 4C illustrates how, in this polymer trap model, metabolic conversion of sucrose and galactinol (MW < 400 Da) to raffinose and stachyose (MW > 600 Da) would lead to a decrease in the osmotic potential within the CC-SE. In support of this model, it has been shown that synthesis of raffinose and stachyose occurs primarily within the intermediary cells (Beebe and Turgeon, 1991; Haritatos et al., 1996). However, the proposed differences in the SEL of plasmodesmata connecting intermediary cells to the bundle sheath and the SE have yet to gain experimental support.

An important argument for symplasmic loading, in addition to the number of plasmodesmata connecting the CC-SE to the surrounding cells, is based on experiments using p-chloromercuribenzenesulfonic acid (PCMBs), a reported membrane-impermeant sulfhydryl-modifying reagent that strongly inhibits sucrose uptake and phloem loading in group II plants by directly blocking the transporter (Delrot, 1987; Bush, 1993). The corollary here is that in group I plants, sucrose uptake and assimilate export should not be, and were not, inhibited by PCMBs (Van Bel et al., 1992, 1993; Flora and Madore, 1996). This has led some workers to conclude that carrier-mediated components are not essential for phloem loading in group I plants. However, given the paucity of our knowledge regarding sugar and/or oligosaccharide carriers in such plants, we cannot discount that the inability of PCMBs to inhibit assimilate export might well reflect the absence and/or inaccessibility of an essential sulfhydryl group on membrane proteins involved in loading or retrieval of these molecules from the apoplasm.

As sugar transporters evolved well before the development of higher plants (Marger and Saier, 1993), it would be surprising, from an evolutionary viewpoint, if in group I plants carriers were completely absent from the plasma membrane of the CC-SE complex, thereby forfeiting the advantages of loading and/or retrieving assimilates from the apoplasm (Fig. 2A). In addition, the observation that many group I plants also transport small to major amounts of sucrose may well indicate the potential operation of two parallel phloem loading systems. Clearly, this is an important area that merits further investigation, as it holds great significance with respect to the molecular and structural evolution of phloem loading mechanisms.

C. Molecular Analysis of Sucrose Carrier(s) Involved in Phloem Loading

Sugar, and in particular, sucrose transport in higher plants has been reviewed quite extensively with respect to the progress made by cloning of cDNAs encoding mono- and disaccharide carriers (Sauer et al., 1994; Tanner and Caspari, 1996; Ward et al., 1997). Therefore, we will focus our attention on
experimental results directly related to phloem loading. Considerable efforts were dedicated to the biochemical isolation of plant sucrose transporter proteins from leaf plasma membrane preparations (Gallet et al., 1989; Lemoine et al., 1989). Indeed, the isolation of a sucrose binding protein from soybean was achieved by Ripp et al. (1988) and its association with CCs was demonstrated by Grimes et al. (1992). However, major advances awaited the application of molecular methods. Important breakthroughs came by using a specifically engineered yeast strain that was defective in all forms of invertase expression. This strain was engineered to metabolize sucrose by introducing a sucrose synthase gene that then allowed complementation cloning of sucrose carriers from spinach and potato (Riesmeier et al., 1993, 1992). Because the genes for the cloned sucrose carriers were highly conserved, the cDNAs for sucrose transporters from spinach and potato (e.g., SUT1) could be successfully used to isolate genes encoding homologous sucrose carriers from Plantago, Arabidopsis and Ricinus (Gahrtz et al., 1994; Sauer and Stolz, 1994; Weig and Komor, 1996).

Studies performed on yeast cells expressing plant sucrose carriers demonstrated that sucrose uptake was inhibited by protonophores and thiol-modifying agents (e.g., PCMBS). In addition, the K_m values for these heterologously expressed sugar carriers were in the range of 1 mM. These functional characteristics were in agreement with earlier results obtained for high affinity sucrose uptake systems studied in planta (see Delrot, 1987; Bush, 1993). Furthermore, injection of SUT1 cRNA and expression in Xenopus oocytes allowed voltage-clamp measurements to be used to analyze the ionic currents that accompany sucrose uptake, via the expressed SUT1 transporter. Findings from such experiments were consistent with the operation of a sucrose-H^+ cotransport system having a functional stoichiometry close to 1:1 (Boorer et al., 1996), values that were in close agreement with earlier studies conducted on plasma membrane vesicles (Bush, 1989).

That these sucrose carriers may indeed play a role in phloem loading was first indicated by in-situ detection of the appropriate mRNA in the minor veins (Riesmeier et al., 1993). Studies employing fusions of the promoter of the sucrose carrier gene to GUS also demonstrated expression within the phloem of leaf tissue (Truernit and Sauer, 1995). In addition, evidence consistent with the functioning of the sucrose carrier in phloem loading was gained by studies conducted on transgenic potato plants in which expression of the sucrose carrier was reduced by expression of antisense constructs under the control of the strong CaMV 35S promoter, or the phloem-specific RolC promoter (Kühn et al., 1996; Lemoine et al., 1996). In both transgenic potato lines, plants displayed symptoms which were considered typical for inhibition of phloem loading: namely, (i) accumulation of high amounts of soluble and insoluble carbohydrates in source leaves; (ii) inhibition of photosynthesis; and (iii) reduced root growth along with a decrease in tuber yield. Reciprocal graft experiments demonstrated that inhibition of sucrose transporter gene expression in leaves was sufficient to produce the above-described symptoms. Interestingly, within individual leaves of such antisense potato plants, mesophyll cells did not respond uniformly to the inhibition of sucrose export: whereas high accumulations of starch was noted in some cells, other cells responded by a reduction in the rate of photosynthesis.

Intriguing questions still remain to be resolved concerning the cellular localization of the machinery which allows loading of assimilates from the apoplasm into the SE. For example, are all the components required for active sucrose uptake located in the SE plasma membrane; i.e., sucrose carriers energized by proton cotransport and H^+-translocating ATPases essential for the establishment and maintenance of the proton motive force? Some insight into this aspect of phloem loading was gained from freeze fracture studies performed on cultured Streptanthus cells, where a high density of globular structures (presumably H^+-ATPases) was found in the SE plasma membrane (Sjölund and Shih, 1983). However, recent immunolocalization studies have indicated that, in Vicia, the H^+-ATPase was more concentrated in the plasma membrane of transfer cells in the minor vein as compared with the SE (Bouche-Pillon et al., 1994). In support of these findings, the c-Myc tagged AHA3 (H^+-ATPase) from Arabidopsis was found to be predominantly associated with the CC plasma membrane (Dewitt and Sussman, 1995). Unfortunately, it is not yet known whether another member of the large AHA gene family is targeted to the SE plasma membrane (11 expressed genes have already been identified in Arabidopsis that encode H^+-ATPases (Dewitt et al., 1996).

Even more complexity is added to the picture when one considers recent immunolocalization studies that have been performed on the sucrose
carrier within the phloem of exporting leaves. Depending on the plant species, sucrose carrier protein has been detected either in the CC, or associated with the plasma membrane of the SE. Using specific antibodies directed against the Arabidopsis sucrose carrier 2 (SUC2) in conjunction with immunofluorescence techniques, SUC2 was identified in CCs in both Plantago and Arabidopsis (Stadler et al., 1995; Stadler and Sauer, 1996, see Fig. 4B). On the other hand, in tobacco, potato and tomato plants, antibodies raised against SUT1 detected the sucrose transporter protein in the plasma membrane of sieve elements in the minor vein of the source leaf and in the petiole phloem (Kühn et al., 1997, see Figs. 4A and 5D,E). Interestingly, the abundance of SUT1 was much higher in the plasma membrane of sieve elements in the petiole as compared to the plasma membrane at the loading sites in the minor vein, suggesting that retrieval into the sieve element along the long-distance transport path is another important function of SUT1. Finally, whether these studies revealed the cellular localization of all sucrose carriers involved in phloem loading in these plants is not yet clear.

In any event, the presence of proteins with high turnover rates in the SE plasma membrane, e.g. SUT1 (Kühn et al., 1997), poses an intriguing problem as to how targeting of membrane proteins may be achieved in these unique enucleate cells of the higher plant phloem. As ribosomes have not been reported from electron microscope-based studies performed on mature (functional) SEs and since Golgi vesicles are also absent from these cells, it would appear that transcription and translation of such proteins may well occur in the CC. Although trafficking of soluble proteins through plasmodesmata into the SE has recently gained experimental support (Balachandran et al., 1997; Ishiwatari et al., 1998), studies have not been performed with hydrophobic integral membrane proteins. Clearly, if these integral membrane proteins are indeed first synthesized in the CC either they must be imported into the SE by some form of specialized chaperone system, or they must be synthesized and inserted into the plasma membrane at the CC orifice of the plasmodesmata. Here again, some form of special mechanism would be required to move the protein(s) along the tortuous pathway into the SE. In this regard, it is interesting to note that earlier studies performed on fluorescently labeled membrane lipids suggested that lipid components may be able to move cell to cell in the ER membrane, whereas lipids in the plasma membrane remained within the target cell (Grabski et al., 1993). Thus, whether the plasma membrane route through the CC-SE plasmodesmata is tenable will require careful experimentation conducted at the CC-SE level.

Finally, in-situ detection of SUT1 mRNA, within the orifice of the CC-SE plasmodesmata (Kühn et al., 1997), as well as in association with the sieve element reticulum (see Fig. 5C), raises the possibility that translation may well proceed within the functional sieve tube system. Clearly, it will be extremely important to determine whether this SUT1 mRNA is indeed translated on special ribosomes located within the sieve tube, or whether these transcripts play another role in the long-distance transport system of the angiosperms.

D. Phloem Loading of Amino Acids

Analysis of phloem sap from a variety of plants has shown that nitrogen is transported almost exclusively in the form of amino acids in sieve tubes (Pate, 1980), with amides such as glutamine and asparagine usually being the most abundant species (Hocking, 1980; Fukumotita and Chino, 1982; Fisher and Macnicol, 1986; Shelp, 1987). Experiments with Ricinus seedlings, which allow control of amino acid supply to the site of phloem loading, showed that glutamine is the preferred amino acid taken up into the sieve tubes (Schober and Konor, 1989; Schober and Konor, 1992). The importance of source metabolism, in determining the mix of amino acid species used for long-distance transport of nitrogen, has been pointed out in other experimental systems. Interestingly, no major differences were found between the content and pattern of amino acids in the leaves and the phloem sap obtained from spinach, barley and sugar beet (Riens et al., 1991; Winter et al., 1992; Lohaus et al., 1994).

Although the functional properties of an amino acid transporter can be studied using a heterologous expression system (Frommer et al., 1994) such as yeast cells, to understand the physiological role of this system requires explicit information on the conditions present within the tissue of interest. Thus, to address the question of which specific transporters are involved in loading amino acids into the CC-SE complex, data will be needed on the actual chemical species and relative concentrations present within the symplasmic and apoplastic compartments at the site of phloem loading. The importance of this
information can be demonstrated by the following example. A transporter that has been characterized (using a heterologous system) as a high affinity cationic amino acid transporter, but which also functions as a low affinity glutamate transporter, could well function as the glutamate carrier, in vivo, because the apoplastic glutamate concentration in the minor vein could be much higher than that for lysine. The problem is therefore to identify the actual concentration experienced by the carriers located in the plasma membranes along the pathway from the mesophyll to the CC-SE complex.

Unfortunately, currently employed techniques do not permit analysis of the various amino acid concentrations present within the cytosol, vacuole and apoplast of specific cells within an intact leaf. The application of in vivo NMR imaging may well prove useful in the resolution of this problem. NMR correlation-peak-imaging was recently successfully applied to the analysis of amino acid and sugar distribution within the hypocotyl of castor bean seedlings. Using this method, Metzler et al. (1995) were able to establish that valine was present only in parenchyma cells of the cortex, whereas glutamine (and/or glutamate) was detected in these same cells as well as in the cells comprising the vascular bundles; lysine and arginine, on the other hand, were present mainly in the vascular tissue. Here we should stress that the finding that valine showed a different distribution from the other amino acids is surprising, because the broad specificity AAPs are able to transport acidic and neutral amino acids, including valine, with similar kinetics. In any event, if NMR imaging techniques can be further refined to provide subcellular resolution, they would provide a powerful method to study amino acid transport at both the cellular and tissue level (see Metzler et al., 1995).

Until such techniques are developed, workers in this field have little choice but to perform experiments that, unavoidably, provide sample averages from an array of cell types within leaf tissues. Nevertheless, the tools for such analyses have become rather highly developed. For example, by using a combination of non-aqueous fractionation, apoplastic washing and laser stylectomy techniques, the sucrose and amino acid composition of the vacuolar, cytosolic, apoplastic and phloem sap could be studied on the same plant (Lohaus et al., 1995). As might well be expected, these studies indicated that the apoplastic concentrations for sugars and amino acids were low in comparison to those present in the cytosol.

However, it is still a matter of some debate as to whether the apoplastic wash technique can provide meaningful information on the actual metabolite concentrations that are present at the site(s) of phloem loading. This concern is heightened by the recent finding that, within the minor veins, phloem loading of sucrose appears to occur across a small fraction of the total surface area (Kühn et al., 1997). In any event, the presence of a large concentration gradient between the phloem sap and the apoplasms would be consistent with active transport of amino acids into the CC-SE complex, a process which may well be mediated by an amino acid-H+ symport system (AAPs).

IV. Regulation of Assimilate Export

There are important questions to address relating to how the plant integrates the overall rate of photosynthesis with the fraction of assimilate production to be exported from the leaf. In relation to this question, it is equally important to understand how the plant assigns priority to the multitude of sinks that will utilize these photoassimilates. This, in its broadest sense, describes the process of assimilate partitioning, and its regulation is critical to the growth, development, and reproductive success of the plant. In essence, mechanisms have to exist which allow the plant to regulate photoassimilate production in autotrophic tissues relative to the requirements of competing sinks (heterotrophic tissues/organs) (Fig. 1A). These mechanisms must function in concert with the processes that operate to control phloem loading rates and relative direction (distribution) of flow, and must accommodate predictable and unpredictable restrictions and demands: i.e., store photoassimilate during the day to accommodate growth and metabolism of sinks during the night; integrate transitions from sink-to-source status in a diurnal or developmental context; respond to changing biotic and abiotic environmental conditions, etc. Clearly, these are highly complex processes that require input from source and sink tissues and may involve responses such as the regulation of transcription and translation, as well as reversible protein modification, etc. Communication is clearly key to the integration of such physiological and/or developmental processes, whether it be from cell-to-cell (i.e., over short distances) or between organs, in the context of a whole-plant response, which involves
the transfer of information over long distances via the phloem and the xylem.

Exciting breakthroughs on some of these communication processes are only now coming to light (see also section II.B). Here, we will discuss models of how plants cells, tissues and organs may use various modes of communication to direct assimilate partitioning and adapt to a wide range of environmental challenges.

A. The Paraveinal Mesophyll—a Model for Regulation of Assimilate Export

Leaves of soybean, as well as a number of other legume and non-legume species, have a specialized mesophyll layer called the paraveinal mesophyll (PVM) (Fisher, 1967). This system will be used to illustrate some important concepts relevant to the intermediate pathway for symplasmic transfer of photoassimilates within the leaf, as well as the interrelationship between specific sources and sink organs. The PVM forms a paradermally (parallel with the epidermal tissue) arranged one-cell-thick reticulum of very large cells that span the interveinal region between the minor veins, and is symplasmically connected to the bundle parenchyma cells at the level of the phloem (Franceschi and Giaquinta, 1983a). In addition, the PVM is symplasmically connected to the cells of the palisade layer and the spongy mesophyll. As illustrated in Fig. 6, the position of the PVM and its symplasmic continuity with the adjacent cell layers may implicate a central role in the regulation of assimilate transfer within the soybean leaf.

With respect to delivery of photoassimilates to the phloem, the laterally oriented, large cells of the PVM appear to play a role in enhancing the efficiency of assimilate transfer from the photosynthetic parenchyma to the phloem, mainly by reducing the number of cell walls (plasmodesmata) that have to be traversed in the pathway, and thus reducing the resistance associated with the symplasmic route. For example, in Vigna radiata, a single PVM cell may span the region between adjacent minor vein phloem, and have 20 or more palisade cells in symplasmic contact (Franceschi and Giaquinta, 1983b). These cells can dramatically reduce the resistance to flow of photoassimilates to the veins for export. In contrast to other cells within the leaf, these cells undergo cytoplasmic streaming (Franceschi et al., 1984) which would be important for mixing and to overcome the diffusional limitations to transport in the cytoplasm of such large cells. Thus, the PVM acts as a high-capacity assimilate collection and transfer network.

This proposed function of the PVM in the soybean leaf was fully supported by $^{14}$CO$_2$ pulse-chase experiments which clearly demonstrated the flux of $[^{14}$C]-photoassimilate through these specialized cells. In addition, morphometric analyses revealed that the number of plasmodesmata that have to be traversed in the photoassimilate transport pathway to the phloem (Lansing and Franceschi, unpub. results). Thus, the pattern and efficiency of assimilate flux from mesophyll cells to the export phloem can be affected by: (i) plasmodesmal frequency, (ii) placement of plasmodesmata, and (iii) cell morphology in relation to the three-dimensional anatomical relationship between various cell layers. Here we should emphasize that the concept of a ‘collecting cell’ network is not new, having been first proposed by Haberlandt (1914). However, the important principles developed by this early German botanist/plant physiologist have often been overlooked in recent studies.

The soybean PVM system also plays an important role in terms of nitrogen assimilate storage, in the form of vacuolar storage proteins (Franceschi et al., 1983; Klauer et al., 1996). Studies on these proteins and nitrogen metabolizing enzymes within the PVM further illustrated that this tissue functions as an important and sophisticated control site for amino acid storage, mobilization and delivery to the phloem long-distance transport system. Experiments investigating the deposition and mobilization of amino acids associated with vacuolar storage proteins indicate the presence of a feedback mechanism that is linked to the nature, or amount, of photoassimilates that are passing through the PVM. For example, the storage proteins accumulate in large amounts in PVM only when nitrogen is not limiting relative to overall growth rate. Subsequently, during seed production, when inorganic nitrogen assimilation is dramatically reduced, the PVM storage proteins are rapidly metabolized, presumably to free amino acids for transport to the developing seeds via the phloem. However, if the plants are continuously depodded, the PVM proteins continue to accumulate to massive levels, even when Rubisco begins to be degraded in the mesophyll cells.
These results are consistent with the notion that the PVM contains some sort of amino nitrogen 'sensor' so that when levels passing through this compartment, in route to the phloem, are higher than the capacity for phloem loading, excess amino acids and storage proteins synthesized from them are transported across the tonoplast for storage in the vacuole. Interestingly, depodding also leads to...
massive buildup of starch in the mesophyll, and this also occurs in the PVM where starch storage rarely takes place (Franceschi and Giaquinta, 1983c). Hence, some form of sugar feedback sensor, with respect to reduced carbon (sugar) build-up, must also reside within these tissues. Collectively, the information obtained on the physiological functions performed by the PVM points to the fact that mechanisms regulating photoassimilate transport out of the leaf exist not only at the site of phloem loading but also in the photoassimilatory cells and the cells that comprise the transfer pathway into the phloem. In this regard the symplasmic route from the mesophyll to the site of phloem loading, via plasmodesmata, may represent an efficient route in terms of the exchange of information molecules that could form part of the sophisticated feedback mechanism(s) which appears to operate between the photoassimilatory cells and the phloem transport system.

B. Plasmodesmal Companion Cell-Mesophyll Communication Network

Although the distribution and frequency of plasmodesmata within the minor veins can vary considerably from species to species, in all plants examined thus far, plasmodesmata interconnect the bundle sheath, phloem parenchyma and CCs. As mentioned previously, there is an expanding body of direct and indirect evidence that supports the hypothesis that plasmodesmata have the capacity to engage in, and regulate, the cell-to-cell trafficking of macromolecules (proteins and protein-nucleic acid complexes). This unique property of plasmodesmata would clearly potentiate supracellular control over tissue function (Lucas et al., 1993). It has also become clear that many plant viruses have acquired the capacity to move from cell to cell by a direct interaction with this plasmodesmal macromolecular trafficking system (Lucas and Gilbertson, 1994; Gilbertson and Lucas, 1996; Ghoshroy et al., 1997). In this regard, Lucas and Wolf (1993) advanced the hypothesis that plant viruses gained this capacity by the acquisition of genes used by the plant to mediate supracellular control over plant development and physiology.

Based on these findings and recent models of plasmodesmal trafficking of information molecules, Balachandran et al. (1995) proposed a novel interpretation for the observation that, when expressed in transgenic tobacco plants, the TMV-MP causes significant changes to carbon metabolism, export and biomass partitioning (Lucas et al., 1993b; Olesinski et al., 1995, 1996). Here it is important to stress that changes in biomass partitioning to the lower stem and root system of these TMV-MP expressing plants required only that the TMV-MP be present in the mesophyll and, further, these effects were shown not to be correlated with MP-induced increase in SEL (Balachandran et al., 1995).

Figure 7 provides a model to account for the TMV-MP results that is based on the concept that plasmodesmata function not only to allow diffusion of metabolites within the symplasm, but also potentiate the selective trafficking of information molecules. Thus, the underlying premise for the model depicted in Fig. 7 is that, under normal physiological conditions, a constant exchange of information molecules occurs between the CC and the mesophyll. These molecules (proteins and/or peptides, protein-mRNA complexes, including short nucleotide sequences, and metabolites) move through plasmodesmata to establish a control network involved in regulating photosynthesis occurring within the mesophyll and photoassimilate (e.g., sucrose and amino acids) loading/export that takes place in the CC-SE complex (see Fig. 7 and Lucas et al., 1996 for full details). Although the sites at which the TMV-MP interacts to induce these changes have yet to be elucidated, the TMV-MP will likely compete with endogenous protein(s) for binding sites that form part of the putative signal transduction cascade. Experimental confirmation of the model presented in Fig. 7 would establish a new conceptual foundation for the study of photosynthesis and carbon partitioning in higher plants.

C. Source-Sink Communication via the Phloem

The above-described local communication network model (Fig. 7) provides a foundation for the study of leaf physiology, but how does the plant coordinate developmental and physiological functions at the whole-plant level? Although it has long been known that plant hormones move within the phloem sap, only recently has it been appreciated that a large number of proteins are also present in this long-distance transport compartment. Based on a thorough study performed on wheat (Fisher et al., 1992), it is clear that a large number of phloem sap proteins move from mature leaves towards the apex, as well as
into strong sink tissues, such as the grain. Furthermore, microinjection studies have demonstrated that phloem sap proteins, collected from squash plants, can undergo cell-to-cell transport through mesophyll plasmodesmata, even when introduced into the target cell at very low levels (Balachandran et al., 1997). The threshold concentration required for plasmodesmal trafficking was estimated to be in the low nM range; i.e., at or below the level required for the action of many plant hormones.

These new findings raise the definite possibility that long-distance signaling, via the phloem, may involve both traditional plant hormones as well as a range of novel macromolecules that have the capability to control either gene expression or enzyme function in distant tissues and organs. Such a long-distance signaling system may well account for the influence of the TMV-MP over resource allocation to the lower stem and roots of TMV-MP expressing tobacco plants. As illustrated in Fig. 7, the TMV-MP expressed within the mesophyll could act on the putative mesophyll input signal ($SM_I$) to alter expression and/or plasmodesmal trafficking of a long-distance signaling molecule ($SM_{id}$) from the CC into the SE.

A rigorous test for this long-distance signaling model will become possible once the proteins and/or nucleic acids within the angiosperm phloem sap become further characterized. Confirmation of this long-distance macromolecular trafficking model would advance our understanding of the manner in which plants operate at a supracellular level (Lucas et al., 1993a). Furthermore, this knowledge would lead to the development of a more complete understanding of the evolutionary processes that gave rise to the enucleate sieve tube system of the angiosperms. Eventually, this information may permit the development of a new generation of plants in which novel controls could be used to manipulate the
delivery of photoassimilates to specific plant organs, such as the grain in the cereals.

V. Conclusions

It has been truly amazing to witness the unfolding of our knowledge regarding the molecular processes engaged in photoassimilate transfer within the leaf from the mesophyll into the phloem. Genes for membrane transport proteins have been cloned which mediate sugar and amino acid uptake from the apoplast into the symplasm and the role of sucrose carriers in phloem loading has been firmly established. In addition, much information has been gained with respect to the structure and function of plasmodesmata. These unique intercellular channels create a cytoplasmic continuity within the plant body which is the hallmark of the supracellular nature of higher plants. Not only do they allow for the symplasmic flow of photoassimilates but, as exciting recent experiments show, they have the capacity to potentiate the movement of endogenous plant macromolecules, proteins and nucleic acids, from cell to cell. We have advanced the hypothesis that the cell-to-cell movement of macromolecules establishes a control network within the leaf which extends to the growing parts of the plant via the phloem, and allows integration of physiology throughout the plant. This hypothesis provides a framework for the study of the diverse and complex interactions between the various cell types involved in photosynthesis, photoassimilate storage and export which transcends the reductionist approach that mere regulation by metabolites will suffice to explain plant growth and development. The challenges ahead will be to devise experiments to test the hypothesis through the identification and characterization of the postulated supracellular regulatory macromolecules.

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Chapter 12

Regulation of Sugar Alcohol Biosynthesis

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Summary 

Although we have long known that sugar alcohols can be important primary photosynthetic products involved in storage and translocation, there has been very little information on gene expression or regulation of enzyme activities associated with metabolism of these compounds. Recent studies, however, indicate that sugar alcohol metabolism is probably as tightly regulated as is conventional carbon metabolism in sink and source tissues. Sugar alcohols have also been demonstrated to be associated with the development of tolerance to drought, salt, temperature, and related stresses, and there is quite limited, but increasing evidence of stress-related regulation of genes and enzymes associated with sugar alcohols. Moreover, several studies of plants transformed with a capacity for sugar alcohol biosynthesis now indicate that these plants have enhanced stress tolerance. All this has important implications for crop improvement and developing understanding of stress tolerance mechanisms in plants.

I. Introduction

Why review sugar alcohols in a volume focused on photosynthesis and regulation? Because sugar alcohols frequently occur in many important crop plants, functioning as primary photosynthetic products and playing major roles in translocation and storage. Sugar alcohols have been overlooked or neglected in the past, due in part to analytical problems, and because they infrequently appear in the staples, e.g., wheat, rice, corn, sorghum, and grain legumes. However, their importance and distribution is now much better recognized, especially in horticultural crops. There are a number of reviews, many on distribution (see Loescher and Everard, 1996, for a listing), some extensive (Lewis and Smith, 1967a,b; Bieleski, 1982; Lewis, 1984), and others quite recent (Stoop et al., 1996). Despite the recent reviews, we really know very little about regulation of sugar alcohol metabolism (see Fig. 1), especially compared to the information on metabolism and regulation of other important plant carbohydrates and related enzymes, e.g., starch and ADPG pyrophosphorylase (Preiss and Sivak, 1996), sucrose and sucrose phosphate synthase (Huber and Huber, 1996), sucrose synthase, and the invertases (Koch, 1996), or even the raffinose-related oligosaccharides (Keller and Pharr, 1996).

Metabolism of sugar alcohols, like other photosynthetic products, must be closely controlled, and it has been reasonable to presume that control is modulated by environmental and developmental factors as well as sink/source interactions. That these controls are manifest at the gene, protein, or enzyme/substrate level is a given, but only a few such studies of sugar alcohol metabolism in higher plants have been completed. It has also been presumed, based until recently almost entirely on correlative evidence in higher plants and more direct evidence from work on algae (Cowan et al., 1992), fungi (Brown, 1978), and lichens (Honegger, 1991), that sugar alcohols play roles in tolerance of environmental stresses, especially those related to cold, salinity, and drought. Several quite recent studies now show that engineering a capacity for sugar alcohol biosynthesis can affect the ability of transgenic plants to withstand salt and drought stress (see below). Since these data confirm roles for sugar alcohols in stress tolerance, it also seems reasonable to presume that stress in turn may profoundly affect partitioning, sink-source transitions, and regulation of these phenomena. Indeed, other quite recent studies show unequivocal and sometimes dramatic effects of stress on gene expression and enzyme activities related to sugar alcohol metabolism. Our focus here is mostly on the straight chain (acylic) polyols. Our goal here is to summarize the recent literature on these compounds and to assess its further implications for regulation.

II. Primary Physiological Roles

A. Photosynthetic Products

Bieleski (1982) has estimated that sugar alcohols account for 30% of global primary production. In algae the sugar alcohol may be the only primary product, but in all sugar alcohol-producing higher plants that have been studied, analyses of $^{14}$CO$_2$ assimilation have usually revealed two major soluble products, the sugar alcohol and the invariably present sucrose. In apple leaves, for example, about 70% of the newly fixed carbon was equally distributed (on a molar basis) between sucrose and glucitol (Grant and ap Rees, 1981). In celery 80 to 90% of the label in mature leaves was recovered as mannitol and sucrose, with about equal amounts of label (on a molar basis) in each (Loescher et al., 1992). Similar results were noted in lilac and apricot, mannitol and glucitol synthases, respectively (Trip et al., 1963; Bieleski and Redgwell, 1977). In species where the galactosyl-sucrose oligosaccharides are significant products (e.g., privet) sucrose and mannitol collectively accounted for less of the total label but were still synthesized in considerable quantities (Loescher et al., 1992). Alternatively, the sugar alcohol may be a secondary photosynthetic product. Hamamelitol, for example, is relatively unimportant initially in Hedera leaves, comprising only a small fraction of the labeled compounds, but it accumulates nonetheless due to slow turnover and little transport (Moore et al., 1997). This labeling pattern is similar to that of several cyclitols, e.g., an extended pulse of $^{14}$CO$_2$ was required for label to first appear and a chase of several days was necessary for label to accumulate in L-quebrachitol in Acer pseudoplatanus (Schilling et al., 1972).
Fig. 1. Schematic diagram showing important features involved in the metabolism and transport of the primary soluble products of photoassimilation (mannitol and sucrose). The information for this diagram comes primarily from work on celery (Apium graveolens var. dulce), but the synthetic pathway has been described in other higher plant species (see text). The diagram illustrates the parallel (and presumably competitive, in terms of substrates) mannitol and sucrose biosynthetic pathways. Various documented (+/−), and possible (?) modulators of the two key regulatory steps [M6PR (2) and SPS; (4)] in the mannitol and sucrose biosynthetic pathways, respectively, are listed. The diagram also illustrates the spatial separation of the anabolic and catabolic pathways for mannitol, and it is the dynamic balance between sink and source activity, translocation between them and sequestration in long and short term storage pools (involving as yet poorly described transport processes) that is ultimately responsible for regulating mannitol pools in the plant. Important enzymatic steps shown in the diagram are: 1) mannose 6P isomerase (note the roles in both anabolic and catabolic pathways); 2) mannose 6P reductase (M6PR); 3) mannitol-1-P-phosphatase; 4) sucrose-phosphate synthase (SPS); 5) sucrose-phosphate phosphatase; 6) sucrose synthase (SS); 7) mannitol-1-oxidoreductase (MTD); 8) hexokinase. Mannitol catabolism involves MTD (7), but alternative degradative enzymes exist in other species, e.g. mannitol 2-dehydrogenase (9) (see Table 1). See also Color Plate 4.
B. Long Distance Translocation

Transport of sugar alcohols has not been systematically investigated except for a study showing that mannitol, glucitol, and galactitol were transported in 57 of more than 500 species (Zimmermann and Ziegler, 1975). Evidence for translocation of other acyclic sugar alcohols is lacking, although the cyclitol inositol may be present in trace amounts in most phloem exudates. Sucrose, however, seems invariably present as a translocated carbohydrate in sugar alcohol-synthesizing higher plants, even when 60 to 90% of the photosynthate consists of a sugar alcohol. Evidence for sugar alcohol phloem transport comes primarily from labeling studies (Webb and Burley, 1962; Bieleski, 1969; Davis and Loescher, 1990; Flora and Madore, 1993), although aphid stylet exudates have been analyzed in at least two reports (Moing et al., 1992, 1997). Knowledge of phloem transport mechanisms is otherwise limited. Most information comes from celery, from which vascular tissues can be isolated relatively easily (Daie, 1987a,b), although glucitol transport has been studied in isolated apple phloem (Bieleski, 1969). Export of both glucitol and mannitol is often related to source synthetic capacity and tissue concentrations (Moing et al., 1994), but in some cases it is not. In olive, for example, stachyose accounted for only 5% of the total leaf label while mannitol accounted for 30%, yet stachyose was the predominant label in phloem sap (Flora and Madore, 1993). Other results suggest that the sugar alcohol is synthesized secondarily (Moing et al., 1992), or is first partitioned into a vacuolar storage pool with sucrose apparently being exported at a faster rate from a transitory (cytosolic) storage pool (Davis and Loescher, 1990).

C. Storage

There is considerable evidence for sugar alcohols as storage compounds. Sugar alcohols often represent the majority of the photosynthetic carbon pools in leaves and other vegetative tissues. There can, however, be distinct differences depending on species, tissue, stage of development, and environmental conditions. Storage in leaves especially depends on the stage of growth. Glucitol in apple seedling and mature apple leaves increased rapidly with maturation, e.g., from 0.9% of dry wt in June to 4.8% in late July, decreasing in August and September, but finally increasing in October, suggesting environmental as well as developmental influences (Whetter and Taper, 1963; Chong, 1971).

Also, although glucitol is the major photosynthetic product and translocate, and is the dominant storage carbohydrate during active growth of sweet cherry, only low concentrations occur in dormant roots, which instead store massive amounts of starch (Keller and Loescher, 1989). However, glucitol becomes a significant component of the xylem-translocated compounds exported from roots to developing shoots in early spring (Loescher et al., 1990). In celery, mannitol synthesized in the mesophyll accumulates temporarily in the petiole parenchyma of mature leaves, being remobilized to the younger (sink) tissues during senescence (Keller and Matile, 1989; Davis and Loescher, 1990). In petiole parenchyma mannitol is stored predominantly in the vacuole (81%), with a lesser amount in the cytosol (19%), but cytosolic concentrations may be quite high (Keller and Matile, 1989). On a shorter time scale, sugar alcohols appear to be a temporary carbon store in source leaf mesophyll tissues, accumulating in the light and being translocated in the dark once sucrose pools, which also perform a temporary storage role, have been depleted (Fellman and Loescher, 1987; Davis and Loescher, 1990). Taken together, all these results not only suggest distinct storage roles for these compounds, but also provide evidence of developmental and tissue specific regulation of acyclic sugar alcohol metabolism, storage, and transport.

Sucrose is more readily metabolized than mannitol in both young and mature celery leaves as well as in sink tissues; mannitol is only utilized in nongreen sink tissues or in young (immature and nonphotosynthetic) leaves before they undergo the sink-source transition in celery (Fellman and Loescher, 1987). Similarly, in olive, despite similar uptake rates of $^{14}$C-labeled fructose, glucose, sucrose, and mannitol in disks from mature leaves, mannitol utilization was barely detectable whereas the sugars were catabolized (Gucci et al., 1996). Hamamelitol turnover in Hedera helix leaves is extremely slow ($t_{1/2}$ ca. 4 years) (Moore et al., 1997). These results demonstrate that the sugar alcohol is metabolically more sequestered than sucrose, and this may have several important physiological implications in storage, translocation, and stress tolerance.

There is now evidence in celery cell cultures and other sink tissues that sequestration or limited metabolism is due at least in part to repression of the mannitol-catabolizing mannitol dehydrogenase.
(MTD) by hexose sugars (Prata et al., 1997). It was already known that glucose-grown cell cultures expressed little MTD activity during active growth, but underwent a marked increase in MTD activity, protein and mRNA upon glucose starvation (Pharr et al., 1995), or when mannitol was the primary carbon source (Stoop and Pharr, 1993). This repression appears to be mediated by hexokinase(s) in a manner comparable to the sugar repression of photosynthetic genes (Stitt et al., 1995; Jang et al., 1997), and these observations are related to the multiple and general effects of tissue carbohydrate status on regulation of gene expression (Koch et al., 1992; Koch, 1996).

D. Roles in Parasitic Plants

Do parasitic plants require special considerations or interpretations of sugar alcohol metabolism? Parasitic species differ widely in their capacity to import and assimilate carbon and inorganic nitrogen. However, all parasites invariably have, compared to their hosts, lower water potentials which are, not surprisingly, accompanied by higher osmolarities and less susceptibility to drought (Stewart and Press, 1990). The osmotic gradient between host and parasite is sometimes due in part to accumulation of organic materials, i.e., glucose, fructose and cyclitols in the mistletoes (Richter and Popp, 1992) and mannitol in many other parasitic species. It is not always clear whether these are derived from their hosts or synthesized by the parasite, but in Orobanche (Harloff and Wegmann, 1993) and perhaps several other genera, e.g., Thesium (Fer et al., 1993; Simier et al., 1993, 1994), this gradient appears to be largely the result of a mannitol synthesizing pathway quite similar to that in celery, i.e., with a mannose 6P isomerase, a mannose 6P reductase, and a mannitol 1P phosphatase all present (Harloff and Wegmann, 1993). It is not yet clear whether these synthetic enzymes are all kinetically similar to those in celery (Simier et al., 1994; A. Fer and P. Simier, personal communication). Orobanche also contains a low affinity mannitol:NAD oxidoreductase (EC 1.1.1.67) (Harloff and Wegmann, 1993). The path for mannitol breakdown appears to depend on the species and involves one of two NAD-dependent enzymes: (1) mannitol 1-oxidoreductase (EC not assigned) (Stoop and Pharr, 1992), or (2) mannitol NAD oxidoreductase (fructose reductase) (EC 1.1.1.67) (Harloff and Wegmann, 1993) (Fig. 1).

Sugar alcohol metabolism occurs in microbial, animal, and fungal systems as well as higher plants. Higher plant metabolism has recently been reviewed (Loescher and Everard, 1996). Here we focus only on those enzymes involved in mannitol and glucitol metabolism (see Fig. 1 and also Table 1) because these sugar alcohols can be primary photosynthetic products, and both have been studied far more extensively than any other sugar alcohol in higher plants.

Mannitol is synthesized in green celery tissues as a result of three cytosolic enzymes (Rumpho et al., 1983; Loescher et al., 1992): (1) mannose 6P isomerase (EC.5.3.1.8), (2) NADPH-dependent mannose 6P reductase (EC.1.1.1.224), and (3) mannitol 1P phosphatase (EC.3.1.3.22) (Fig. 1).

\[
\text{Fructose 6P} \rightarrow \text{Mannose 6P} \rightarrow \text{Mannitol} \rightarrow \text{Mannitol} \]

Glucitol is similarly synthesized via a NADPH-dependent glucose 6P reductase (aldose 6P reductase, EC. 1.1.1.200) (Hirai, 1981; Negm and Loescher, 1981) and a sorbitol (glucitol) 6P phosphatase (EC. not assigned) (Grant and ap Rees, 1981). Mannitol may be synthesized in Orobanche either by a mannose 6P reductase or perhaps by a low affinity mannitol 1P oxidoreductase (fructose 6P reductase) (EC. 1.1.1.17) (Harloff and Wegmann, 1993).

The path for mannitol breakdown appears to depend on the species and involves one of two NAD dependent enzymes: (1) mannitol 1-oxidoreductase (EC. not assigned) (Stoop and Pharr, 1992), or (2) mannitol NAD oxidoreductase (fructose reductase) (EC.1.1.1.67) (Harloff and Wegmann, 1993) (Fig. 1).

\[
\text{Mannitol} + \text{NAD}^+ \rightarrow \text{Mannose} + \text{NADH} \\
\text{Mannitol} + \text{NAD}^+ \rightarrow \text{Fructose} + \text{NADH} \\
\]

Glucitol is apparently primarily metabolized by an NAD-dependent sorbitol (glucitol) dehydrogenase (EC 1.1.1.14) (Negm and Loescher, 1979), but other
Table 1. This table lists the known synthetic, degradative and selected ancillary enzymes associated with acyclic sugar alcohol metabolism in higher plants. Also listed are EC numbers (where assigned), the source, and accession numbers if they have been cloned from higher plants. Please note sorbitol is synonymous with glucitol. Further information is given in the footnote.

<table>
<thead>
<tr>
<th>Name, synonyms and abbreviation</th>
<th>EC Number</th>
<th>Organism</th>
<th>Accession number</th>
<th>Reference if cloned</th>
</tr>
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<tr>
<td>Synthetic Enzymes</td>
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<tr>
<td>NADP-dependent sorbitol 6P dehydrogenase;</td>
<td>1.1.1.200</td>
<td><em>Eriobotrya</em></td>
<td>D11080</td>
<td>Hirai, 1981 (p)</td>
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<tr>
<td>NADPH-dependent aldose 6P reductase;</td>
<td></td>
<td><em>Malus</em></td>
<td></td>
<td>Negm and Loescher, 1981 (p)</td>
</tr>
<tr>
<td>A6PR</td>
<td></td>
<td></td>
<td></td>
<td>Kanayama et al., 1992 (c)</td>
</tr>
<tr>
<td>mannose 6P reductase;</td>
<td>1.1.1.224</td>
<td><em>Apium graveolens</em> var. dulce</td>
<td>U83687</td>
<td>Loescher et al., 1992 (p)</td>
</tr>
<tr>
<td>M6PR</td>
<td></td>
<td></td>
<td></td>
<td>Everard et al., 1997 (c)</td>
</tr>
<tr>
<td>mannotol 1P:NAD oxidoreductase</td>
<td>1.1.1.17</td>
<td><em>Orobanche</em></td>
<td></td>
<td>Harloff and Wegmann, 1993 (p)</td>
</tr>
<tr>
<td>NADPH-dependent aldose reductase; galactitol synthesising aldose reductase</td>
<td>unassigned</td>
<td><em>Euonymus japonica</em></td>
<td></td>
<td>Negm, 1986 (p)</td>
</tr>
<tr>
<td>NADPH-dependent ribose 5P reductase</td>
<td>unassigned</td>
<td><em>Adonis</em></td>
<td></td>
<td>Negm and Marlow, 1985 (p)</td>
</tr>
<tr>
<td>sedoheptulose reductase; ketose reductase; SedR</td>
<td>unassigned</td>
<td><em>Primula</em></td>
<td></td>
<td>Häfliger et al, 1998 (p)</td>
</tr>
<tr>
<td>Synthetic and Degradative Enzymes</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>NAD-dependent polyol dehydrogenase; ketose reductase; sorbitol dehydrogenase; L-iditol:NAD 5-oxidoreductase/dehydrogenase; SDH</td>
<td>1.1.1.14</td>
<td><em>Malus</em></td>
<td>*</td>
<td>Negm and Loescher, 1979 (p)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Zea mays</em></td>
<td></td>
<td>Doehlert, 1987 (p)</td>
</tr>
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<td></td>
<td></td>
<td><em>Glycine max</em></td>
<td></td>
<td>Kuo et al., 1990 (p)</td>
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<tr>
<td>Degradative Enzymes</td>
<td></td>
<td></td>
<td></td>
<td>Wanek and Richter, 1993 (p)</td>
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<tr>
<td>mannitol dehydrogenase;</td>
<td>unassigned</td>
<td><em>Apium graveolens</em> var. rapaceum and dulce</td>
<td>U24561</td>
<td>Stoop and Pharr, 1992 (p)</td>
</tr>
<tr>
<td>mannitol: mannose 1-oxidoreductase; MDH; MTD</td>
<td></td>
<td></td>
<td></td>
<td>Williamson et al., 1995 (c)</td>
</tr>
<tr>
<td>NADP-dependent sorbitol dehydrogenase</td>
<td>unassigned</td>
<td><em>Malus</em></td>
<td></td>
<td>Yamaki, 1984 (p)</td>
</tr>
<tr>
<td>sorbitol oxidase</td>
<td>unassigned</td>
<td><em>Malus</em></td>
<td></td>
<td>Yamaki, 1980 (p)</td>
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### Chapter 12  Regulation of Sugar Alcohol Biosynthesis

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>EC</th>
<th>Plant Name</th>
<th>Gene Reference</th>
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<tr>
<td>mannitol NAD:oxidoreductase; mannitol 2-dehydrogenase; fructose reductase</td>
<td>1.1.1.67</td>
<td>Orobanche</td>
<td>*</td>
</tr>
<tr>
<td><strong>Associated Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mannose 6P isomerase; phosphomannose isomerase; phosphohexomutase; PMI</td>
<td>5.3.1.8</td>
<td>Aiptum graveolens var. dulce</td>
<td>*</td>
</tr>
<tr>
<td>mannitol 1P phosphatase</td>
<td>3.1.3.22</td>
<td>Aiptum graveolens var. dulce</td>
<td>Orobanche</td>
</tr>
<tr>
<td>sorbitol 1P phosphatase</td>
<td>not assigned</td>
<td>Malus</td>
<td></td>
</tr>
<tr>
<td>glyceraldehyde 3P:NADP reductase; non-reversible or non-phosphorylating triosephosphate dehydrogenase; GNR</td>
<td>1.2.1.9</td>
<td>Aiptum graveolens var. dulce</td>
<td>*</td>
</tr>
<tr>
<td><strong>Function undefined</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH-aldose reductase; AR</td>
<td>1.1.1.21</td>
<td>Glycine max</td>
<td>R23901</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hordeum vulgare</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromus inermis</td>
<td></td>
</tr>
</tbody>
</table>

* indicates where the corresponding genes have been cloned from sources other than higher plants. By using EC numbers (where available) in conjunction with the web site “Enzymes” (http://teosinte.agron.missouri.edu/enzyme.html) access to all clone and sequence information currently available on public data bases can be obtained (via the associated “hot links”). The letters in parentheses following references indicate whether the study covers the protein and enzyme activity (p), or molecular cloning (c).
enzymes may be important in some situations (Yamaki, 1982; Yamaki and Ishikawa, 1986; Moriguchi et al., 1990) (See Table 1).

Important to regulatory questions are recent advances in what we know of these enzymes at the gene and protein level. Sequences have been available for a number of sugar alcohol related enzymes in animal and microbial systems, and now at least three higher plant enzymes have been cloned and sequenced: the aldose 6P reductase from apple (Kanayama et al., 1992), a celery mannitol 1-oxidoreductase (Williamson et al., 1995), and a celery mannose 6P reductase (Everard et al., 1997) (Table 1).

M6PR and A6PR are closely related, with their deduced amino acid sequences showing 64% identity and 84% similarity. Considerable homology also exists between these proteins and the mammalian ALR2 class of aldose reductases which are NADPH-specific, monomeric 35kD proteins (see Section VIII.A). The monomeric masses of A6PR and M6PR, either directly measured (Loescher et al., 1992; Kanayama and Yamaki, 1993) or from the deduced amino acid sequences (Kanayama et al., 1992; Everard et al., 1997), are 35 kD. However, gel filtration chromatography indicated the native molecular masses of A6PR and M6PR to be 65 kD (Hirai, 1981) and 58 kD (Loescher et al., 1992), respectively, suggesting that they may be homodimers. Whether this indicates a genuine difference between the plant and animal aldose reductases or is a result of anomalous behavior of the plant enzymes during gel filtration needs to be resolved.

Why aren’t the isomerases, mutases, epimerases, and phosphatases important regulatory factors? Two studies (Grant and ap Rees, 1981; Loescher et al., 1992) suggest that the mannose 6-phosphate isomerase and the glucitol 6P and mannitol 1P phosphatases are possible, but unlikely candidates for several reasons. The hexose phosphates, fructose 6P, glucose 6P, and mannose 6P are in equilibrium in celery leaf tissues, extractable activities of these enzymes are all far in excess of flux rates through the biosynthetic pathways, and the pool sizes of the phosphorylated hexitols (the last intermediate in the biosynthetic pathway) are all very small.

IV. Developmental Regulation and Primary Roles

Studies of sink-source transitions can provide excellent insight into the relative roles that specific enzymes and processes play in plant metabolism (Turgeon, 1989). However, only a few such studies are known in sugar alcohol-synthesizing plants, and these have focused on either glucitol or mannitol metabolism where they usually involved the enzymes aldose 6P reductase (A6PR) and sorbitol (glucitol) dehydrogenase (SDH) or mannose 6P reductase (M6PR) and mannitol 1-oxidoreductase (MTD), respectively. The results have invariably indicated that sugar alcohol metabolism is generally under very tight developmental control. For example, sink-source interconversion studies of apple and celery leaves show that developing leaves undergo transitions from sinks with high rates of sugar alcohol oxidation to sources with reduced or negligible capacity for sugar alcohol degradation and increased capacities for synthesis. These transitions are accompanied by increases in the corresponding synthetic enzymes. A6PR increases in glucitol-synthesizing apple leaves (Loescher et al., 1982; Yamaki and Ishikawa, 1986) and cotyledons (Yamaki, 1980; Kanayama et al., 1995) and developing peach leaves (Merlo and Passera, 1991). Similar changes occur in M6PR and SPS in mannitol- and sucrose-synthesizing celery leaves (Davis et al., 1988). Data from young celery leaves are also consistent with enzyme assays that show little detectable M6PR in young leaves and non-green tissues. Other evidence shows that as leaves undergo the sink-source interconversion there are corresponding decreases in degradative enzymes (Loescher et al., 1982; Fellman and Loescher, 1987; Merlo and Passera, 1991).

Lewis (1984) has reviewed other reports comparing synthesis and degradation that show that the ability to synthesize, accumulate, and degrade sugar alcohols can vary tremendously among different plant tissues during development within a given plant tissue and between otherwise closely related taxa. Sinks, for example, have little or no capacity to synthesize sugar alcohols. Apple root tips, bark and wood of stems, and root tissues can synthesize only a little glucitol from fructose (Grant and ap Rees, 1981). Celery root tips can accumulate and metabolize mannitol, but do not synthesize mannitol from other sugars (Stoop and Pharr, 1992). Celery petioles, however, which contain some green tissues, can synthesize mannitol, although at lower rates than leaves (Keller and Matile, 1989). Similarly, although glucitol is commonly translocated in many Rosaceous species, it is found in only trace amounts in nectar.
These nectaries rapidly convert glucitol to glucose, fructose, and even sucrose, but apparently cannot convert these sugars to the hexitol (Bieleski and Redgwell, 1980).

Unfortunately, very little else has been done to correlate activities of the sugar alcohol enzymes with development of either photosynthetic (source) competence, storage or sink activity. However, more comprehensive studies of gene regulation are now possible, and there has been progress. Genes for several of the key anabolic and catabolic enzymes have recently been cloned. In celery, for example, M6PR gene expression and enzyme activity are now known to be limited to green tissues and to be under tight transcriptional regulation during leaf initiation, expansion and maturation. These data confirm a close relationship between development of photosynthetic capacity, mannitol synthesis, and M6PR activity (Everard et al., 1997). Another study (Prata et al., 1997) of MTD gene expression and enzyme activity suggests that mannitol oxidation is transcriptionally regulated in celery suspension cultures (a model sink tissue). Transcriptional regulation appears to be controlled by endogenous carbohydrate levels by mechanisms like those involved in repression of photosynthetic gene expression via hexokinase (Sheen, 1994). However, there are as yet no data to show how the synthetic and degradative pathways are coordinated.

V. Localization of Synthetic and Degradative Steps

Both indirect (Lewis, 1984) and direct immunolocalization (Everard et al., 1993) evidence now leads to the conclusion that sugar alcohol biosynthesis is predominantly cytosolic, and not associated with vacuoles, chloroplasts, mitochondria, peroxisomes, or membranes, although these same studies suggest that M6PR may also be present in the nuclear compartment. Both A6PR and M6PR genes appear to lack signal peptides, and there is no evidence that the M6PR peptide is post-translationally processed in vivo (J. D. Everard, unpublished), further confirming the cytosolic location. As the key step in mannitol biosynthesis, M6PR is also predominantly found in green spongy and palisade parenchyma of leaf mesophyll tissues and bundle sheaths (Everard et al., 1993), but not the vascular parenchyma. This contrasts with galactinol synthase, which immuno-localization suggests is located in the intermediary cells of the phloem of cucurbit source leaves, where it may facilitate the phloem loading of galactosyl-sucrose oligosaccharides like raffinose (Beebe and Turgeon, 1992).

Consistent with these immunocytochemistry results in both mannitol and sucrlosyl oligosaccharide synthesizing species, labeling studies in olive (Olea) (Flora and Madore, 1993) suggest spatial separation of stachyose and mannitol biosynthesis, with relatively rapid mannitol synthesis occurring within the photosynthetic mesophyll tissues and slower raffinose synthesis occurring closer to, and probably within, the minor veins. Somewhat similar differences in labeling of mannitol and stachyose were noted in privet (Ligustrum, also in the Oleaceae), but no differences between sucrose and mannitol labeling were observed in celery which does not transport or synthesize raffinose oligosaccharides (Loescher et al., 1992).

There is no recent or immunocytochemical evidence suggesting that sugar alcohol biosynthesis is chloroplastic, but targeting a bacterial enzyme for mannitol biosynthesis to that compartment in transgenic plants has some implications for stress tolerance (see Section VII.E). The mannitol degrading enzyme MTD is also cytosolic, and is found in various sink tissues, i.e., meristems of celery root apices, in young expanding leaves, in the vascular cambium, and in the phloem, including the parenchyma and the sieve-element/companion cell complexes (Zamski et al., 1996). The association between MTD and phloem has been suggested as a means by which mannitol may reenter the metabolically active carbohydrate pool and thus provide energy to support phloem function (Zamski et al., 1996). This is analogous to the proposed role of phloem associated sucrose synthase (Geigenberger et al., 1993; Nolte and Koch, 1993) (Fig. 1).

VI. Membrane Transport

Single saturable, or biphasic, active, PCMB-sensitive, turgor-sensitive specific mannitol uptake systems have been described in celery storage parenchyma (Keller, 1991). Keller speculated that the mannitol carrier was at the plasmalemma and involved in active proton-cotransport. Similar results were reported for glucitol transport in apple fruit tissue protoplasts and isolated vacuoles, where
transport was facilitated by a PCMB, CCCP, and orthovanadate sensitive saturable carrier on the plasmalemma (Yamaki and Asakura, 1988) and a somewhat similar ATPase-coupled carrier on the tonoplast (Yamaki, 1987). Studies of older apple fruit tissue discs indicated that this system was also turgor sensitive (Berüter, 1993). Berüter also concluded from compartmental analyses that the cytosol was part of the apparent free space, although this was dependent on osmotic strength of the bathing medium and the age of the tissue. Sugar alcohol transport in these sink systems appears to have some characteristics common to those reported for sucrose in sink tissues (Patrick, 1993). In isolated celery phloem preparations (Daie, 1986; Daie, 1987a,b) and olive leaf disks (Flora and Madore, 1993), however, mannitol uptake appeared to be insensitive to PCMB, perhaps because the mannitol carrier does not always have an exposed sulfhydryl group as part of its active site. Alternatively, in species lacking PCMB sensitivity, anatomical studies suggest that phloem loading may be symplastic (Turgeon and Beebe, 1991; van Bel et al., 1992).

Another option is that phloem loading and uptake of mannitol in source tissues may involve a distinctly different carrier from that in sink tissues. Recent work (Salmon et al., 1995) with plasma-membrane vesicles (PMVs) from phloem strands isolated from mature celery petioles or from mature petioles devoid of vascular bundles showed that the PMVs from phloem strands had a higher ATPase activity suggesting the possibility of different carriers. Further work supported this by showing that PMVs from phloem tissues accumulated mannitol and sucrose in response to an artificial proton-motive force, in agreement with the existence of proton/substrate carriers, but PMVs from petioles devoid of vascular bundles accumulated only mannitol under the same conditions. Similarly, modeling of glucitol and sucrose export from mature leaves suggested that glucitol and sucrose export capacities involved different transporters with different kinetics, i.e., glucitol transport was saturated at normal photosynthetic rates resulting in glucitol accumulating during the day while sucrose content appeared to be maintained at a constant level (Moing et al., 1994). More recently, Moing et al (1997) reported that glucitol concentrations in the leaf were quite high and not significantly different from the phloem concentrations, thus enough for symplastic loading; however, transport of both glucitol and sucrose was inhibited by PCMB, an indicator of apoplastic loading (van Bel et al., 1994).

In some bacteria, specific phosphorylase-linked permeases for mannitol, glucitol, or galactitol transport are well characterized. The mtl operon, for example, consists of a number of genes, including a permease and a mannitol IP dehydrogenase (Weng and Jacobson, 1993; Henstra et al., 1996), but no equivalent system has been reported for sugar alcohol transport in higher plants. On the other hand, Bieleski and Redgwell (1980), studying secretion in Prunus nectaries, noted that glucitol transport (perhaps as an integral part of phloem unloading) was associated with conversions to fructose and glucose. Similarly, Yamaki (1982) reported evidence in apple for a tonoplast-associated glucitol oxidase or a cytosolic glucitol dehydrogenase (Yamaki and Asakura, 1988) that may be involved in glucitol transport and conversion, resulting in hexoses accumulating as the predominant carbohydrates in fruit vacuoles.

Another consideration involves inorganic nutrition. Although boron has historically been considered a phloem-immobile element, recently Brown and coworkers (Hu et al., 1997) successfully isolated and characterized boron-sugar alcohol complexes from the phloem sap of celery and the extrafloral nectar of peach. In celery they were able to directly analyze phloem sap by matrix-assisted laser desorption-Fourier transform mass spectrometry, with verification by HPLC and gc-ms (Penn et al., 1997). Their data revealed that B is present in the phloem as the mannitol-B-mannitol complex. Molecular modeling further predicted that this complex is present in the 3,4 3´,4 bis-mannitol configuration. In the extrafloral nectar of peach, B was present as a mixture of glucitol-B-glucitol, fructose-B-fructose, or glucitol-B-fructose. These findings provide a mechanistic explanation for the observed phloem B mobility in these species and associated susceptibility to B deficiency (Hanson, 1991), and they may also have further implications for sugar alcohol transport.

VII. Secondary Physiological Roles

Immobility means that plants must respond to environmental changes, and carbohydrate-regulated genes represent an especially valuable mechanism for acclimation (Koch, 1996). If it is a reasonable presumption that environmental stresses affect regulation of sugar alcohol metabolism, then there
should be examples of stress affecting sugar alcohol synthesis, breakdown, storage, transport, partitioning, and sink-source transitions. Such changes leading to accumulation and/or synthesis may be evident at the whole plant level, but others could be quite subtle, e.g., evident only in changes in cellular compartmentation. Although evidence of stress effects on sugar alcohol metabolism is limited compared to that available for some other carbohydrates, the results taken as a whole are clear and convincing.

A. Roles as Compatible Solutes and in Osmoregulation

Stresses that drastically alter cellular water potential, e.g., salinity, drought, and cold stresses, can also specifically cause a number of compounds to accumulate (Schobert, 1977; Pollard and Jones, 1979; Yancey et al., 1982; Wyn Jones, 1984; Popp, 1984; Bonnett and Incoll, 1993). These compounds, which include both acyclic and cyclic sugar alcohols, the imino acid proline, quaternary ammonium compounds (e.g., glycine betaine), and tertiary sulfonium compounds, were originally thought to act primarily as osmotic regulators, by maintaining ‘osmotic balance’. But other compounds, both inorganic (K⁺, Cl⁻, Na⁺, SO₄²⁻) and organic (hexose reducing sugars), can play roles in osmotic adjustment (Munns and Weir, 1981; Sharp et al., 1990), and it is now understood that the first group of compounds may have other protective qualities. This change in view came from work on sugar-tolerant yeasts which accumulate nonreducing sugars (trehalose) and sugar alcohols (including glycerol) in response to osmotic stress. From this latter work the term ‘compatible solute’ was first introduced (Brown and Simpson, 1972).

Data on compatible solutes comes primarily from studies in which high concentrations did not interfere with in vitro enzyme activities (Flowers et al., 1977; Ahmad et al., 1979; Pollard and Jones, 1979; Smirnoff and Stewart, 1985; Manetas et al., 1986; Karsten et al., 1996), and sometimes even protected them from deleterious effects of salt (Ahmad et al., 1979; Manetas et al., 1986) or heat (Paleg et al., 1981; Smirnoff and Stewart, 1985). Compatible solutes also protected the cellular transcriptional apparatus from perturbations by salt in vitro (Gibson et al., 1984) and increased thermal tolerance of isolated chloroplast thylakoids (Seemann et al., 1986). However, for compatible solutes to perform in vivo like they do in vitro, they would seem to have to accumulate to very high concentrations, e.g., up to 500 mM. Indeed, many of the compounds listed above do achieve these critical levels if asymmetric compartmental distribution is assumed. But, in transgenic plants where the capacity to synthesize these compounds has been engineered, concentrations of the compatible solute may be well below 100 mM (a value achieved only by assuming that all accumulation occurs in the cytosol). Protective effects have nonetheless been demonstrated, and the term ‘osmoprotectant’ has been coined to accommodate these observations (Bohnert and Jensen, 1996).

Several biophysical roles have been proposed for sugar alcohols and other compatible solutes (e.g., proline) and their interactions with biological structures (Galinski, 1993). In the first, they may substitute for water in protein hydration shells and membrane structures or, at least in the case of proline, protect exposed hydrophobic domains of proteins and thus maintain conformation at water activities that otherwise cause dehydration and denaturation (Schobert, 1977; Crowe et al., 1988). A second alternative suggests uneven distribution of compatible solutes in a protein solution where compatible solutes are excluded from protein hydration spheres; in some way bulk water structure is ordered so that biological structures become preferentially hydrated and conformation is maintained as the bulk water activity is otherwise lowered. There are data supporting both hypotheses, i.e., direct interactions (Webb and Bhorjee, 1968) or exclusion (Timasheff, 1993). In the third and perhaps supplemental protective role, proline and sugar alcohols may scavenge hydroxyl radicals, protecting enzymes from denaturation during stress (Smirnoff and Cumbes, 1989; Jennings and Burke, 1990; Popp and Smirnoff, 1995) (see also Section VII.E).

B. Cold Hardiness

As compatible solutes, sugar alcohols could play roles in cold tolerance and freezing resistance. Cold injury often results from desiccation due to extracellular freezing and consequent withdrawal of water from the protoplast by the growing ice crystals. Work on insects strongly suggests a role in cold hardiness (Storey and Storey, 1991), especially now that there is a glucitol related gene associated with cold acclimation (Niimi et al., 1993). Trends in woody plants may be analogous to insects, but despite
numerous efforts to demonstrate such relationships the mostly correlative evidence is less than convincing. There is little evidence of other than passive increases in sugar alcohols that could be explained by reduced demand and decreased export due to low temperatures, there are no compartmentation studies, there are as yet no cold regulated genes associated with sugar alcohol metabolism in higher plants, and there are no transgenic plants with improved cold tolerance resulting from sugar alcohol accumulation.

For example, Sakai (1960), studying 19 woody species with a range of sugar alcohol-synthesizing capabilities, found no direct correlation between sugar alcohol content and cold hardiness; however, later work showed increases in both sugar alcohols and raffinose in response to cold stress (Sakai, 1966). Some apple tissue culture experiments suggested that using glucitol as a carbon source increased cold tolerance (Pieniazek et al., 1978). In other work with xylem sap extracted from apple shoots, glucitol increased with leaf senescence and low (0 to 4°C) temperatures. As temperatures continued to fall during dormancy, glucitol levels generally increased, with concentrations fluctuating sharply with changes in temperature (Williams and Raese, 1974; Ichiki and Yamaya, 1982). Although xylem sap contained 10 to 12 times as much glucitol in December and January as compared with October, the amount of glucitol in the entire tissue remained fairly constant throughout dormancy except for a slight increase during December and January. Exposing apple shoots to near zero temperatures could increase sap glucitol as much as 40-fold within 24 h (Williams and Raese, 1974). Thus, some increases in sap concentrations may indeed be related to changes in tissue distribution (and perhaps cellular compartmentation) rather than synthesis.

Similar changes in sap glucitol were found in sweet cherry (Prunus avium), with the highest levels following exposure to subfreezing temperatures in January. As in apples, overall tissue glucitol content increased little, but there were increases in xylem sap levels. Later in the season, however, these changes were not correlated with decreasing temperatures: although total carbohydrate levels rose, glucitol levels declined, and reducing sugars increased until just before full bloom where they may have been important in early bud development (Sauter and Ambrosius, 1986; Loescher et al., 1990).

Further evidence of relationships between cold hardness and sugar alcohols in deciduous perennials is limited, and interpretations are complicated by studies where unidentified ‘carbohydrates’ are correlated with hardiness (Flinn and Ashworth, 1995). Hirai (1983), however, reported glucitol content and activity of the glucitol synthesizing enzyme (A6PR) to increase in late autumn and decrease in spring in leaves of loquat (Eriobotrya, a subtropical evergreen). Similar results were obtained with seedlings in controlled environments where activity was increased by low temperature. In winter, in bark of the evergreen olive, mannitol levels were more than double what they were in the summer (Drossopoulos and Niavis, 1988). Studies of low temperature acclimation in several cacti (Opuntia) species showed, depending on the tissue, from 2× to over 9× increases in soluble sugars, including a nearly 3× increase in mannitol in the hardiest species (Goldstein and Nobel, 1994).

As already mentioned, there are no cold regulated genes associated with sugar alcohol metabolism. There may, however, be several cold regulated genes similar to genes related to sugar alcohol metabolism. For example, cDNA of an aldose reductase-related gene has been isolated from ABA-treated bromegrass cell suspension cultures during the induction of freezing tolerance (Lee and Chen, 1993b). It appears that both aldose reductase-related mRNA and enzyme activity were elevated during ABA-induced hardening in these cell suspension cultures; however, polyethylene glycol treatments (to mimic dehydration) did not elicit similar effects (Lee and Chen, 1993b).

C. Drought Stress

Very few studies focus specifically on acyclic polyols and drought stress, and interpretations of these and other stresses may be confounded by confusing primary and secondary effects. Osmotic adjustment generally does not occur without a decrease in growth rate (Munns, 1988), and photosynthetic products could accumulate due to reduced sink demand resulting from stress-induced growth inhibition, in the same way that low temperature-induced accumulations could also be explained by reduced demand and decreased export from source leaves (Popp and Smirnoff, 1995). Also, it is not always clear whether osmotic adjustment is due to active synthesis and accumulation of solutes or passive due to leaf dehydration and shrinkage (Hsiao, 1973). Nonetheless, glucitol was the dominant soluble
carbohydrate and its content doubled in drought-stressed *Prunus* (Ranney et al., 1991). In apple, water stress resulted in preferential accumulation of glucitol and glucose instead of sucrose and starch (Wang et al., 1995; Wang et al., 1996). In *Fraxinus* mannitol not only changed significantly in leaves throughout the diurnal cycle, but also played a large role in the seasonal osmotic adjustment which occurred in response to summer drought (Marigo and Peltier, 1996). More recent comparisons of drought stressed and control trees and seedlings showed that these acclimated to quite low predawn leaf water potentials (–4 MPa and lower) without apparent wilting or cessation of photosynthesis. Drought resulted in very high mannitol and malate concentrations, i.e., calculated at 280 and 600 mM, respectively, while sucrose remained relatively low, less than 30 to 50 mM (Guicherd et al., 1997). Similar recent studies in olive showed that both mannitol and glucose collectively accounted for approximately 30% of the leaf’s osmotic potential, and studies of salt stress showed that both mannitol and the mannitol to glucose ratio increased rapidly, but it was not clear how these related to drought stress. Cytosolic concentrations were, however, calculated to be adequate for mannitol to be effective as a compatible solute (Gucci et al., 1997).

Despite the limited studies of acyclic polyols, there are numerous examples of drought-induced accumulation of other soluble carbohydrates (for a review see Popp and Smirnoff, 1995). In systems involving cyclic polyols, pinitol increased five-fold in drought stressed *Cajanus* (Keller and Ludlow, 1993), in *Artemisia* accumulations of a cyclitol, probably quebrachitol (Schilling et al., 1972), were clearly drought related (Evans et al., 1992), and in *Pinus pinaster* pinitol doubled when the osmotic potential of the nutrient solution was lowered (Nguyen and Lamont, 1988). In systems involving sucrose there is evidence that water-stress may generally result in higher sucrose and lower starch levels than in non-stressed leaves (see Zrenner and Stitt, 1991, and papers cited therein).

In alternative approaches using transgenic plants, two recent studies have shown that plants with genetically modified capacities for soluble carbohydrate biosynthesis were more drought tolerant. Engineering tobacco plants for biosynthesis of trehalose, a non-reducing disaccharide, greatly increased their capacity to survive drought, but phenotype was altered and growth rate was decreased 30–50% (Holmström et al., 1996). Similar work was done with transgenic tobacco plants that had the capacity to accumulate bacterial fructans (polyfructose). The transgenic plant grew significantly better than controls under polyethylene glycol-mediated drought stress, growth rates were higher, as were fresh and dry weight yields. Weight differences were observed in all organs and were particularly pronounced in roots. However, unlike the trehalose-synthesizing transgenic tobacco plants, under unstressed control conditions the presence of fructans had no significant effect on phenotype, growth rate, and yield (Pilon-Smits et al., 1995).

Work with other presumptive compatible solutes has shown distinct changes in gene expression with drought stress. Proline is one of the most common compatible osmolytes in water-stressed plants (Taylor, 1996), and several studies have shown drought related changes in gene expression, i.e., activation of proline biosynthesis and inactivation of degradation. For example, a nuclear gene for mitochondrial proline dehydrogenase is upregulated by proline but downregulated by dehydration (Kiyosue et al., 1996). There is also induction (osmoregulation) of the proline synthesizing pyrroline-5-carboxylate reductase gene with either seed maturation or salt stress (Verbruggen et al., 1993), and there are salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases (Rentsch et al., 1996).

Although no sugar alcohol related plant genes have been studied in the context of drought stress, expression of a barley gene homologous to aldose and aldehyde reductases has been found that is restricted to the embryo and temporally correlated with acquisition of desiccation tolerance (Bartels et al., 1991; Roncarati et al., 1995). Aldose reductase may also be present in corn seedling tissues which can utilize glucitol as a carbon source (Swedlund and Locy, 1993), and there is evidence in soybean seedlings which appear to have a functioning glucitol pathway of both aldose and ketose reductase activities (Kuo et al., 1990). As already mentioned, however, although Lee and Chen (1993b) were able to increase cold hardness using polyethylene glycol for osmotic stress treatments, there was no detectable aldose reductase mRNA accumulation in bromegrass suspension cultures.

### D. Salt Stress

The correlation between salinity stress and sugar
alcohol accumulation in higher plants is quite strong. Such studies represent not only the majority of the publications on stress and sugar alcohol accumulation, but also where the most progress has been made. The first work was on glucitol metabolism in Plantago (Ahmad et al., 1979; Lambers et al., 1981) and then later in numerous other species (Gorham et al., 1981; Briens and Larher, 1982; Everard et al., 1994; Stoop et al., 1994; Popp and Smirnoff, 1995; Tattini et al., 1996) which showed that acyclic polyols increased substantially with salt stress. There are similar studies of the cyclic polyols (Popp and Smirnoff, 1995). All of this is further supported by the literature documenting osmoregulatory roles for polyols in marine algae (Kirst, 1990). However, like much of the algal literature, most of the higher plant literature is correlative, and the mechanisms involved have not been studied. There are exceptions: mechanisms leading to glycerol accumulation in the euryhaline alga Dunaliella have been studied extensively (Cowan et al., 1992). If we accept that sugar alcohols function as compatible solutes or osmoprotectants in transgenic plants (Tarczynski et al., 1993; Thomas et al., 1995) as apparently do glycine betaine (Nomura et al., 1995) and proline (Kishor et al., 1995), the conclusion that accumulation is an adaptive response is compelling.

The literature on sugar alcohols and salt stress in higher plants often presents interpretational problems. Other compatible solutes, e.g., glycine betaine (Rhodes and Hanson, 1993), proline (Delauney and Verma, 1993), and the cyclic polyols (Popp, 1995; Popp and Smirnoff, 1995), are synthesized in response to stress and are not significant products under non-stressed conditions. However, in higher plants the acyclic polyols are, as already discussed, often primary photosynthetic products. Accumulation may, therefore, represent a consequence rather than an adaptation to stress, the result of suppressed growth and/or reductions in tissue water content. Differential changes between the sugar alcohols and other photosynthetic products (e.g., sucrose and starch) may also be misleading due to the relatively sequestered nature (in terms of their capacity to be metabolized) of polyols (Trip et al., 1964; Fellman and Loescher, 1987; Gucci et al., 1996). For example, passive accumulation of mannitol in response to salt stress has been reported for olive. Mannitol increased in olive leaves in response to salinity stress, but the magnitude of the increase had little relationship to the intensity of the salt treatment or the salt tolerance of the cultivar or species. Indeed, in olive increases in mannitol accumulation (concentration) could result from a decrease in tissue water content (Tattini et al., 1994; Gucci et al., 1997a; Gucci et al., 1997b; Tattini pers. comm.) without any change in carbon partitioning or mannitol metabolism, but other data suggest that mannitol actively accumulates with salt stress (Gucci et al., 1998). Since starch is frequently depleted under salt stress (Everard et al., 1994; Tattini et al., 1996), data on tissue pool sizes should be supplemented with measures of carbon flux into these various carbon pools to understand the metabolic processes underlying accumulation.

Studies of celery stressed with sodium chloride (Everard et al., 1994) or excess macronutrients (Stoop and Pharr, 1994a,b) have shown that stress effects on mannitol accumulation were due to changes in both carbon partitioning and utilization. These changes can be linked to regulation at both the enzyme and gene level (Everard et al., 1994; Stoop and Pharr, 1994b; Loescher et al., 1995; Pharr et al., 1995; Williamson et al., 1995; Stoop et al., 1997). For example, in newly fully expanded leaves of celery plants subjected to 300 mM NaCl carbon flux into mannitol was maintained at control rates despite a 70% decrease in overall carbon assimilation. Maintaining mannitol synthesis came at the expense of sucrose, and the ratio of label in mannitol to sucrose increased four-fold. Associated with this shift was a two fold increase in extractable M6PR activities. However, salt-induced increases in M6PR extractable activity were not associated with a concomitant rise in M6PR protein which suggests post-translational enzyme regulation (Everard et al., 1994).

In developmental studies, as photosynthetic capacity increased and as leaves developed and matured, more carbon was partitioned into mannitol with proportionately less into sucrose, and these changes were accompanied by increased activities of enzymes in the mannitol biosynthetic pathway (Davis et al., 1988). With salt stress, however, mannitol biosynthesis was further enhanced in younger leaves, with 40% of the carbon partitioned into mannitol at 300 mM NaCl compared to 10% in controls. This change in carbon fixation was mostly at the expense of sucrose (Loescher et al., 1995). This salt enhanced increase in mannitol partitioning was accompanied by a six-fold increase in extractable M6PR activity (Everard et al., 1994). Recent results (J. D. Everard, unpublished) show that these increased M6PR
activities were associated with concomitant increases in M6PR transcript and protein levels, indicating salt-induced transcriptional control. Such results are certainly consistent with other observations of salt-induced gene expression (Bohnert et al., 1995; Cushman and Bohnert, 1995; Deutch and Winicov, 1995). See chapters in this volume on CAM for other examples.

On a whole plant basis, mannitol pool sizes are dictated by a balance between synthesis and degradation, processes that are spatially and developmentally separated in celery (Figure 1) and also presumably influenced by long distance transport as well as salinity stress. Although nothing is yet known about mannitol transport in salt stressed plants, considerable information is now available on degradative processes and their regulation. Just as M6PR and subsequent mannitol synthesis and storage in source tissues are enhanced by salt stress, salt induced decreases in mannitol degradation and subsequent accumulation in sink tissues are due to a down regulation of mannitol catabolism via the enzyme MTD (Stoop and Pharr, 1994b; Williamson et al., 1995; Stoop et al., 1996).

E. Free Radical Scavengers

Drought and salt stress are closely related. Indeed, imposition of salt stress may generally be related to a two phase response (Munns et al., 1995) with the first involving a large decrease in growth rate caused by the salt outside the roots, i.e., an osmotic response with decreases in stomatal conductance. In the second phase there could be a salt-specific response to toxic effects within the plant. Thus, in the first phase decreased stomatal conductance could increase production of free radicals and the damage associated with photoinhibition. In this context there is evidence in fungi and other organisms that sugar alcohols like glucitol and mannitol and cyclic polyols like myo-inositol serve a protective function as free radical scavengers (Smirnoff and Cumbes, 1989; Jennings and Burke, 1990), but most of this evidence has been in vitro. There is also evidence that an ability to deal with these active oxygen species may be important in dessication tolerance (Smirnoff, 1993). However, in vivo data are quite limited. With no data available for higher plant systems, the beneficial effects for stress tolerance have been controversial (Munns, 1993). Recently, however, tobacco was transformed with a bacterial mannitol 1P dehydrogenase targeted to the chloroplast by the addition of an amino-terminal transit peptide (Shen et al., 1997). These transgenic tobacco plants accumulated mannitol from 2.5 to 7 μmol/g fr wt. One transgenic line accumulated approx 100 mM mannitol in chloroplasts and was identical to the wild type in phenotype and photosynthetic performance. The presence of mannitol in chloroplasts resulted in an increased resistance to methyl viologen (MV)-induced oxidative stress, documented by the increased retention of chlorophyll in transgenic leaf tissue following MV treatment. It is especially important to note that mannitol did not reduce production of reactive oxygen species, but it did apparently provide additional protection beyond that normally present in nonstressed plants.

Alternatively, in a sequencing study of the mannitol degrading MTD from celery a protein database search has revealed that the ELI3 pathogenesis-related (PR) proteins from parsley and Arabidopsis are also very closely related to MTD, and treatment of celery cells with salicylic acid resulted in increased MTD activity and mRNA (Williamson et al., 1995). This has led to the conclusion that increased MTD activity may provide an additional source of carbon and energy for response to pathogen attack (Williamson et al., 1995; Stoop et al., 1996). Since mannitol is a scavenger of active oxygen species, it may also be likely that MTD by decreasing mannitol levels enhances a plant’s defensive arsenal which often includes pathogen-induced production of active oxygen species (Sutherland, 1991). These MTD data provide additional support for mannitol as an important component of a plant’s response to divergent types of stress.

VIII. Regulation at the Molecular Level

As is clearly evident from the numerous examples above, both developmental and environmental factors affect expression of sugar alcohol related genes and extractable activities of sugar alcohol related enzymes. In other systems and organisms, enzyme activation or inactivation or modulation of enzyme kinetics by oxidation and reduction of enzyme thiols or by protein phosphorylation is common, as is sensitivity to substrates and activators. Unfortunately, no enzyme involved in sugar alcohol metabolism is as well characterized in terms of its regulation as any of those involved in the parallel pathways of sucrose metabolism, i.e., phosphorylation and dephos-
phorylation of SPS (Huber and Huber, 1996) and sucrose synthase (Huber et al., 1996) or the translational mechanisms related to expression of the invertases (Koch, 1996). We are unaware of any data to suggest that any of the sugar alcohol-related enzymes are phosphorylated or dephosphorylated as part of a post-translational regulatory mechanism, nor is there as yet any evidence for a role for fructose 2,6-bisphosphate which is a factor in several other aspects of carbohydrate metabolism (Stitt, 1990). It remains to be determined if these and other mechanisms are involved in regulation of sugar alcohol metabolism in higher plants; however, some data are quite suggestive.

A. Evidence from Other Systems and Organisms

In the alga Tetraselmis (Platymonas) subcordiformis, regulation of mannitol levels during osmotic acclimation appears to depend on enzyme properties rather than on gene activation. The key and apparently irreversible synthetic step is a mannitol 1-phosphate dehydrogenase, which exhibits increased activities in the presence of high NaCl concentrations. In contrast, the degradative pathway starts with a mannitol dehydrogenase (a 2-oxidoreductase) that is sensitive to NaCl. Such results suggest that mannitol levels are directly regulated via alternative pathways, with activities differentially sensitive to NaCl (Richter and Kirst, 1987). Regulation of glycerol content in the algal Dunaliella demonstrates similar features: pathways with enzymes with irreversible actions with different substrate affinities and pH optima for the backward and forward reactions (Kirst, 1990; Cowan et al., 1992).

Animal systems may provide some important clues. Sequence analysis of cDNA clones showed M6PR to be closely related to A6PR from apple (Everard et al., 1997). The analysis also showed both M6PR and A6PR to be members of the aldose reductase ALR2 division (EC 1.1.1.21) of the aldo-keto reductase super-family. This family of monomeric NADPH-specific oxidoreductases has been described extensively in mammalian systems at the gene (Bohren et al., 1989; Carper et al., 1989), enzyme (Wermuth, 1985) and protein structure levels (Wilson et al., 1992). In mammals, these aldose reductases play a central role in osmotic adjustment in renal inner medulla cells (Burg, 1995) and are almost certainly associated with the etiology of the disabling complications associated with diabetes in several tissues (Kador and Kinoshita, 1985). Recent evidence shows mammalian aldose reductases to be transcriptionally regulated in response to osmotic perturbations (Ferraris et al., 1994; Burg, 1995), and in the context of stress tolerance mechanisms and osmoregulation, osmotic (tonicity) responsive elements (promoters) have been identified (Ferraris et al., 1994; Ruepp et al., 1996; Daoudal et al., 1997).

Aldose reductase-like proteins and genes have been identified in higher plants, but for the most part their physiological roles remain undefined. However, two have been shown to be stress induced (Bartels et al., 1991; Lee and Chen, 1993b; Roncarati et al., 1995), and promoter analysis has identified motifs that confer the developmental expression observed in vivo and perhaps the responses to ABA (Roncarati et al., 1995). As already discussed (Section VII.D), transcription of M6PR is induced in young celery leaves under salt stress and MTD is repressed, but a description of the mechanisms involved must await further analysis of the flanking regions of genomic clones.

Despite similarities to the mammalian aldose reductases, M6PR and A6PR differ from their counterparts in their substrate specificities, i.e., requiring aldose phosphates instead of aldoses although the mammalian enzymes may utilize sugar phosphates (Srivastava et al., 1982). Sequence analysis indicates that the residues lining the putative sugar substrate pocket differ considerably, which may explain the substrate differences; however, those amino acids involved in holding NADPH in position are fully conserved in both plant and animal enzymes (Wilson et al., 1992; J. D. Everard, unpublished). The mammalian enzymes are also generally much less selective than M6PR and A6PR, with an array of aromatic and aliphatic carbonyl compounds as other effective substrates. The $k_m$ for glucose (the substrate leading to glucitol accumulation in kidneys and other tissues subjected to hyperglycemia) is $9 \times 10^2$ times higher than that for DL-glyceraldehyde, the usual experimental substrate (Morjana et al., 1989).

In animals, large changes in cellular redox states do not occur normally in most tissues and there is also little evidence for reversible enzyme S-thiolation in the regulation of metabolic reactions (Ziegler, 1985). Nonetheless, post-translational redox mediated regulation of the mammalian aldose reductases has been extensively reported as causing changes in their
activation state and their sensitivity to aldose reductase inhibitors (Vander Jagt et al., 1990; Petrash et al., 1992; Bohren and Gabbay, 1993). Phosphorylation is apparently not a factor, and disulfide bridges are not involved (Grimshaw et al., 1989; Borhani et al., 1992). Nonetheless, redox regulation likely involves mixed sulfides which have been implicated in modifying the activation state and inhibitor sensitivity in vitro (Bhatnagar et al., 1989; Cappiello et al., 1996) and in vivo (Cappiello et al., 1995; Grimshaw and Lai, 1996).

Redox modification is certainly important in plants, especially during short-term adaptation of various photosynthetic enzymatic activities (Scheibe, 1994), and it may also be a factor in sugar alcohol biosynthesis which is often closely linked to photosynthesis. In chloroplasts, in the light, electrons are transferred to thioredoxin via ferredoxin and the reduced thioredoxin then modulates the activities of key enzymes (Buchanan, 1980; Ziegler, 1985; Scheibe, 1991; Scheibe, 1994). This is a common regulatory mechanism in chloroplasts where redox levels vary enough during a diurnal cycle to enable disulfide linkages to form reversibly in the presence of a suitable catalysts (i.e., the thioredoxins). But, both M6PR and A6PR are cytosolic (see Section V), and the majority of redox-regulated plant enzymes are plastidic (Scheibe, 1994). Very few redox-regulated extra-plastidic plant enzymes have been identified (Florencio et al., 1993; Anderson et al., 1995). Also, the biochemical requisite for reductive (and/or oxidative) modification reactions is the presence of regulatory sequences carrying cysteine residues which are subject to reversible redox changes (Scheibe, 1991; von Schaewen et al., 1995). For example, differences in cysteine content apparently distinguish between a redox sensitive chloroplastic glucose-6-phosphate dehydrogenase (G6PDH) and an insensitive cytosolic G6PDH in potato (von Schaewen et al., 1995). Although M6PR and A6PR contain cysteines, four and five respectively, the apparently critical cysteine residue (cys-298) that is sensitive to redox regulation in mammalian aldose reductases (Bohren and Gabbay, 1993; Cappiello et al., 1996) is missing from both M6PR (Everard et al., 1997) and A6PR (Kanayama et al., 1992) as well as two plant aldose reductases (Bartels et al., 1991; Lee and Chen, 1993a). On the other hand, many sugar alcohol metabolizing enzymes in plants are sensitive to sulfhydryl reagents and oxidation (Ikawa et al., 1972; Negm and Loescher, 1979; Negm and Loescher, 1981; Negm, 1986; Stoop et al., 1995, 1998). M6PR is particularly sensitive: high levels of DTT are essential to maintain enzyme activity during extraction (Loescher et al., 1992).

There are examples of redox related mechanisms of enzyme regulation that are distinct from those involving sulfhydryl residues. Many enzymes are sensitive to NADP/NADPH ratios. For example, in fungi, where mannitol is not only common but often so abundant that it is the common currency of carbon metabolism (Lewis and Smith, 1967a), a number of specific synthetic and catabolic fungal enzymes have been identified, e.g., various mannitol and mannitol 1-phosphate dehydrogenases that are responsible for polyol and sugar interconversions. Some of these enzymes, e.g., mannitol dehydrogenase (mannitol: NADP 2-oxidoreductase, EC 1.1.1.138), are apparently very sensitive to in vitro alterations in the NADP/NADPH ratio (Ruffner et al., 1978), leading to the proposal that small changes in the physiological NADP and NADPH concentrations, i.e., small changes in the redox state of the coenzyme, would have a considerable effect on the rate of mannitol oxidation (Morton et al., 1985). Other similar enzymes, however, were not sensitive to changes imposed by growth on some substrates (Singh et al., 1988) which argues against the hypothesis that in fungi these enzymes are involved in meeting or responding to cellular demands for NADPH (Niehaus and Jiang, 1989). However, pH effects and unresolved compartmentation questions may confound these analyses for both forms of the coenzyme as well as the putatively sensitive enzymes (and their isozymes).

B. Sources and Recycling of Reductant

The NADPH necessary for sugar alcohol biosynthesis is apparently supplied by the non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (E.C. 1.2.1.9). This cytosolic enzyme is found in all photosynthetic organisms except cyanobacteria (Mateos and Serrano, 1992; Michels et al., 1994). It has been proposed that this enzyme is involved in a photosynthetic shuttle transferring reducing power as NADPH from the chloroplast to the cytosol (Kelly and Gibbs, 1973b). In support of its role in sugar alcohol biosynthesis GAPDH was found in celery (Rumpho et al., 1983) at levels substantially higher than in several species.
that do not synthesize sugar alcohols (Kelly and Gibbs, 1973a). Whether this enzyme contributes to regulation of sugar alcohol biosynthesis awaits further evaluation of its kinetic parameters and substrate sensitivities in sugar alcohol synthesizing species. In non-sugar alcohol synthesizing spinach, however, the in vivo concentrations of substrates, cofactors, and products suggest that the GAPDH reaction is distant from thermodynamic equilibrium and is regulated, i.e., inhibited primarily by the product NADPH (Scaglia et al., 1990; Trost and Pupillo, 1993). Demand for NADPH in sugar alcohol biosynthesis via M6PR and subsequent phloem export of the sugar alcohol from the leaves could therefore modulate GAPDH activity and in conjunction with Pi levels also modulate the 3-PGA/G3P shuttle and photosynthesis.

In the context of NADPH export, sugar alcohol biosynthesis may have the same implications for maintaining a high photosynthetic capacity as does activity of the mitochondrial electron transport chain in the light (Krömer, 1995). Photosynthetic rates in many sugar alcohol producing plants are high compared to ‘typical’ C3 species. Celery, for example, has been reported to have a very high photosynthetic rate and to differ from typical C3 species in having a low CO2 compensation point (Fox et al., 1986). High photosynthetic rates (for a C3 species) have been confirmed, but anatomical and immunocytochemical studies and reevaluations of gas exchange in celery rule out the usual CO2 concentrating mechanisms and the unusually low CO2 compensation point (Everard et al., 1994; W. Loescher, unpublished).

The hypothesis that sugar alcohol synthesis may provide further use for reductant is not without precedent. For example, respiration of glucose through the alternative oxidase in Plantago coronopus roots was diverted into glucitol synthesis when these tissues were exposed to NaCl (Lambers et al., 1981). By so doing the plants diverted carbon and utilized excess energy, normally dissipated through the alternative oxidase, to accumulation of a compatible solute (glucitol) associated with enhanced salt tolerance in this and other species (see Section VII.D). Jennings and Burke (1990) have also proposed that the cycling of carbon, and hence reducing power, through sugar alcohol synthesis and degradation may provide a pathway by which fungi ‘spill’ energy during periods of stress.

IX. Conclusions and Considerations for Future Research

Our understanding of sugar alcohol metabolism, particularly of the enzymes and a few of the genes related to glucitol and mannitol, has only recently begun to progress. As can be seen from this discussion, we are beginning to develop some insights into the biochemical, transcriptional and post-transcriptional control of biosynthesis and degradation of mannitol. Although transgenic glucitol synthesizers are also available (Tao et al., 1995; Sheveleva et al., 1998), little is known about glucitol regulation, and nothing is known about regulation of other sugar alcohols found in higher plants. We know very little about intracellular transport (which relates to compartmentation issues) or the mechanisms related to intercellular or long-distance transport (which relates to phloem and partitioning issues) for any of the sugar alcohols. The systems involved are complex for two major reasons. The need to regulate carbon partitioning in plants has been discussed in detail (Stitt et al., 1987), and all the arguments for the need to regulate carbon flow in starch and sucrose synthesizers are equally relevant to sugar alcohol synthesizers, with the added complications of another synthetic pathway with one or more critical regulatory steps competing for the same pools of substrates and intermediates (Fig. 1). If we accept the increasing evidence that sugar alcohols have a role in stress tolerance in higher plants, this role certainly requires further modulation of metabolism, storage, and transport. In finding answers to these problems, we will not only develop insight into regulation of carbon partitioning and crop productivity, but also into some mechanisms of stress tolerance. As others have argued (Bartels and Nelson, 1994; Stitt and Sonnewald, 1995; Bohnert and Jensen, 1996), use of transgenic plants and the results of even seemingly minor improvements in stress tolerance could have major implications for plant breeding and crop improvement.

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Summary

Roughly ten percent of higher plant species possess a mechanism for reserve carbon allocation based on soluble fructose polymers (vacuolar fructan), which differs markedly in its enzymology, subcellular compartmentation and regulation from the more common starch-based carbon economy. This includes many economically important species, especially the temperate grasses and cereals. This chapter describes these novel elements associated with fructan metabolism, with particular emphasis on fructan synthesis in photosynthetic tissue.

Fructan structures, although based on variations in a few characters (polymer size, glycosidic linkage, etc.) are shown to be both varied and complex, differing markedly between species but possessing a consistency within species which argues for a biosynthetic mechanism with a high degree of specificity. The enzymological mechanisms currently thought to be involved are discussed, with particular reference to the involvement of multifunctional enzymes and the strong effects of both substrate and enzyme concentration on the chemical nature of the products in vitro. The enzymatic polymerization of authentic grass fructan has been achieved, but the conditions required in vitro do not coincide with those expected in the vacuole, the currently accepted site of synthesis. The properties of fructosyl transferases in general are shown to be unusual and we emphasize the need to reconcile the characteristics of enzymes in vitro with the patterns of metabolism and conditions observed in the tissue.
The regulation of fructan synthesis is discussed in relation to the pivotal role of sucrose as the sole substrate and as the key element in the coarse control of fructan accumulation, apparently acting at the level of gene expression and de novo enzyme synthesis. Sucrose mediated feedback inhibition of starch metabolism via phosphate transport does not apparently occur in grass leaves. This isolation of chloroplast metabolism from cytosolic sucrose accumulation indicates a fundamental difference in the fine control of central carbon metabolism between fructan and starch accumulators.

Recently, non-fructan, starch-accumulating plants such as maize, spinach and tobacco have been transformed with bacterial genes for fructan synthesis and shown to accumulate fructan. The current value of such transgenics is in terms of what they may tell us about the regulation of primary carbon metabolism in the recipient plants. Transgenics currently provide little insight into the nature or control of endogenous fructan metabolism. Some neglected aspects of the physiology these transgenics are considered by comparison with endogenous reserve carbon metabolism in untransformed plants.

### I. Introduction

A number of recent reviews have considered the structure, metabolism and physiology of fructans (oligo- and poly-fructosyl sucrose) in higher plants (Wiemken et al., 1995; Pollock et al., 1996; Pollock and Kingston-Smith, 1997). There is interest in these compounds in terms of their agricultural or industrial potential (Fuchs, 1993a,b), and their metabolism is of general scientific relevance because of a) the discontinuous occurrence of the syndrome within the plant Kingdom; b) its unusual cellular location; c) its distinctive mechanism of synthesis and d) its close interrelationship with sucrose metabolism. The metabolic analysis of fructan metabolism in higher plants began in the 1950s, culminating in the publication of a generic theory of fructan enzymology by Edelman and Jefford in 1968. Subsequently, this theory has been tested in a range of accumulating species and modifications proposed. Sucrose has been confirmed as the major substrate but in addition, appears to have significant regulatory roles (Pollock et al., 1996). This chapter considers the current status of models for both the metabolism of fructans and its regulation, with particular reference to leaves, where synthesis and breakdown are linked closely to photosynthetic carbon metabolism. Comparisons are drawn with the fine control of sucrose metabolism in non-fructan plants.

Enzymes and genes associated with bacterial fructan synthesis have been available for many years and have now been expressed in a number of non-fructan accumulating plants to provide alternative sinks for the perturbation of whole plant carbon balance (Ebskamp et al., 1994; van der Meer et al., 1994; Röber et al., 1996). Genes for a few plant fructosyl transferases have recently become available (Sprenger et al., 1995). This chapter considers the ways in which such novel materials have increased our knowledge of both fructan and sucrose metabolism in plants.

### II. The Distribution and Structure of Fructan

Roughly 10% of the higher plant flora exhibit fructan metabolism (Hendry and Wallace, 1993). Most of the experimental work has concentrated upon the economically important groups: the Asterales (e.g. Jerusalem artichoke and chicory); the Gramineae (mainly C₃ temperate members of the Poales including wheat, barley and temperate forages); and the Liliales (e.g. onion, leek and garlic). Within these families comparatively few species have been studied in great detail, but it has become very clear, following the development of modern separative and analytical techniques, that the range of diversity within fructan structures is very large. The most common form of fructan in higher plants is based upon the sequential addition of fructosyl residues to a sucrose motif, leading to a polydisperse, water soluble, non-reducing polymer containing one glucose residue per molecule of fructan (Pollock et al., 1996, and references therein). There have been reports of fructan series which do not contain glucose residues, but these constitute only a minor component in vivo. (Ernst et al., 1996). The complexity of fructan structures arises in four ways via; a) variation in polymer length, b) variation in the glycosidic linkage between adjacent
fructan residues, c) branching of linear chains, and d) through the position of the sucrose motif in the sugar chain. At its simplest, common in members of the Asterales, the sucrose motif is terminal and the polymer is an homologous series with the type structure G-1,2F-1,2-(F)n, with adjacent fructose residues linked in the β orientation (Edelman and Jefford, 1968). Where n = 1, the trisaccharide is known as 1-kestose. This trisaccharide appears to be present in all species which accumulate fructans, even in species such as the forage grasses, where the predominant polymer is of β-2,6 conformation. In Jerusalem artichoke, the fructan (also termed inulin) chain is linear, every member of the series between n = 1 and n = ca 40 can be isolated, and there is approximately the same mass of fructans in each separable oligosaccharide (i.e. molar abundance declines with increasing size; Edelman and Jefford, 1968).

In other species the situation is more complex. Two other trisaccharides have been isolated; 6-kestose (G-1,2-F-6,2-F) and neokestose (F-2,6-G-1,2F). In the latter, chain elongation from both fructose residues with both linkage patterns is observed, leading to structures which are branched, mixed-linkage or contain an included glucose residue (Fig. 1). The variation in mean polymer size between species is very large (Table 1) but tends to be constant within species. Size may also be affected by the extent to which environmental conditions promote fructan accumulation. What is also consistent within species is the relative abundance of specific oligosaccharide isomers. Not all grasses accumulate all the possible structural variants and the ‘fingerprints’ produced by thin-layer chromatography (compare tissue extracts in Fig. 2) or by high-performance anion-exchange chromatography are very distinctive (Pollock and Cairns, 1991; Chatterton et al., 1993; Slaughter and Livingston, 1994). In temperate grasses, the high molecular weight polymer (which will not pass through dialysis membrane: also termed levan) is almost entirely a linear β-2,6 linked fructan with a terminal glucose (Pollock et al., 1979; Figure 3), but analysis of the smaller fructan oligosaccharides in Lolium temulentum showed a progressive decline in the proportion of included glucose residues and 2:1 linkages as the mean size of the oligosaccharides increased (Table 2).

Isotopic tracer studies (Cairns and Pollock, 1988a; Sims et al., 1993) have demonstrated unequivocally that sucrose is the major donor of fructose residues to growing fructan chains in vivo and that 1-kestose is the first labeled intermediate. Subsequently it appears that fructan residues are transferred to a range of oligosaccharide acceptors, most, if not all, of which are in isotopic equilibrium. The pattern of these acceptors is species-specific and changes radically with increasing size to give a much simpler ‘final’ structure.

A fuller discussion of this complexity can be found in Pollock et al., (1996), but the brief outline given above raises a number of interesting issues in terms of the regulation of the process and the species-specificity of the structures synthesized. Fructans appear unique among storage polymers in terms of the precision of the biosynthetic process within species and its variability between them. This species-specificity can be reproduced in vitro (Figs. 2 and 3) and implicates structural differences in the enzymes from different species as the primary determinant of the structure of the fructan product (Cairns and Ashton 1993). Any complete mechanistic hypothesis for the enzymology of fructan synthesis will need to explain these properties.

III. Physiology and Enzymology of Fructan Metabolism

A. Fructan Synthesis in Storage Roots

A previous review (Cairns, 1993) summarized earlier physiological and enzymological studies in roots, tubers and bulbs. These studies, particularly those of inulin metabolism in tubers of H. tuberosus by Edelman and co-workers (summarized in Edelman and Jefford 1968) provided both the core model and terminology currently used for the enzymes of fructan synthesis in plants. Briefly, this model designates two monofunctional enzymes: a) sucrose:sucrose fructosyl transferase (SST) which forms 1-kestose by fructosyl transfer from donor to acceptor sucrose, and b) fructan: fructan fructosyl transferase (FFT) which reversibly elongates acceptor fructans by the transfer of one fructosyl residue, at the expense of the equivalent shortening of a donor fructan, to produce a linear, β-2,1 linked polymer (i.e. FFT catalyses no increase in the number of glycosidic bonds and hence no net synthesis of fructan). The energy conserved in the glycosidic bond of sucrose is nearly as high as that in uridine diphosphoglucose (28 vs. 32 kJ mol⁻¹; Dey, 1980) and is sufficient to
<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>G$\leftrightarrow$F</th>
<th>Sucrose (ubiquitous)</th>
<th>G$\leftrightarrow$F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisaccharides</td>
<td>G$\leftrightarrow$F1F</td>
<td>F$\leftrightarrow$G$\leftrightarrow$F</td>
<td>neokestose</td>
</tr>
<tr>
<td>1-kestose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all accumulators</td>
<td>Poales</td>
<td>Liliales</td>
<td>6-kestose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poales</td>
<td>Liliales</td>
</tr>
<tr>
<td>Tetrasaccharides</td>
<td>G$\leftrightarrow$F1F1F</td>
<td>F1F$\leftrightarrow$G$\leftrightarrow$F</td>
<td>G$\leftrightarrow$F</td>
</tr>
<tr>
<td>1,1 nystose</td>
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<td></td>
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<tr>
<td>Asparagus</td>
<td>6</td>
<td>Asparagus</td>
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<tr>
<td>all accumulators</td>
<td>F</td>
<td>F</td>
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<tr>
<td></td>
<td>6</td>
<td>Wheat</td>
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<td></td>
<td>Poales</td>
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<td></td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. temulentum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentasaccharides</td>
<td>G$\leftrightarrow$F(F)$_2$1F</td>
<td>F$\leftrightarrow$G$\leftrightarrow$F1F</td>
<td>G$\leftrightarrow$F</td>
</tr>
<tr>
<td>all accumulators</td>
<td>F1F$\leftrightarrow$G$\leftrightarrow$F1F</td>
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<td></td>
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<tr>
<td>Asparagus</td>
<td>6</td>
<td>Asparagus</td>
<td>6</td>
</tr>
<tr>
<td>L. temulentum</td>
<td>(F)$_2$</td>
<td>Oat</td>
<td>F</td>
</tr>
<tr>
<td>L. temulentum</td>
<td>Oat</td>
<td>Oat</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td></td>
<td>Wheat</td>
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<tr>
<td>L. temulentum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G$\leftrightarrow$F1F</td>
<td>F$\leftrightarrow$G$\leftrightarrow$F</td>
<td>G$\leftrightarrow$F1F</td>
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<td>F</td>
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<td>F</td>
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<tr>
<td>Wheat</td>
<td>L. temulentum</td>
<td>Wheat</td>
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</tr>
<tr>
<td>Oat</td>
<td>L. temulentum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. The complexities of structure observed in tri-, tetra-, and pentasaccharides isolated from various monocot species. The ‘minimalist’ structural conventions suggested by Pollock are adopted:

(G$\leftrightarrow$F = Sucrose; F$\leftrightarrow$G$\leftrightarrow$F = Neokestose;
F1F = two adjacent residues linked $\beta$2,1;
F = two adjacent residues linked $\beta$2,6 (from Lewis, 1993).
6
F
Table 1. Variation in mean molecular size of fructans from different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean molecular size (kDa)</th>
<th>Method employed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gramineae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phleum pratense</em></td>
<td>42</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td><em>Phleum pratense</em></td>
<td>46</td>
<td>End group analysis</td>
</tr>
<tr>
<td><em>Lolium perenne</em></td>
<td>12</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td><em>Festuca pratensis</em></td>
<td>12</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td><em>Dactylis glomerata</em></td>
<td>20</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td><em>Bromus inermis</em></td>
<td>5</td>
<td>End group analysis</td>
</tr>
<tr>
<td><strong>Liliales</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Allium sativa</em></td>
<td>9</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td><strong>Asterales</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Helianthus tuberosus</em></td>
<td>5</td>
<td>End group analysis</td>
</tr>
</tbody>
</table>


Table 2. Linkage analysis of pooled high degree of polymerization fructans from *Lolium temulentum* following fractionation by GPC

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Degree of Polymerization (DP)(^a)</td>
<td>7.2</td>
<td>12.8</td>
<td>30.1</td>
</tr>
<tr>
<td>Ratio of 2,6- to 2,1-linkages(^b)</td>
<td>2.4:1</td>
<td>12.6:1</td>
<td>40.1:1</td>
</tr>
<tr>
<td>Ratio of terminal to linkage Glc residues(^c)</td>
<td>2.6:1</td>
<td>7.4:1</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Mean DP was estimated from the Glc:Fru ratio.
\(^b\) The ratio of 2,1- to 2,6-linkages was estimated from the relative peak intensities of m/z = 161, 190 and 162, 189, respectively.
\(^c\) The ratio of terminal glucose to linkage glucose was estimated from relative peak areas following GC separation of partially methylated alditol acetates.
\(^d\) Linkage glucose peak too small for estimation.

Source: Sims et al. 1992

Fig. 2. Qualitative analysis by TLC of the fructan products of a 10h incubation of sucrose with enzyme from leaves of *Lolium temulentum*, (Lt), barley (B), oat (O) and wheat (W). Products of the enzyme (E) are compared with the native water soluble carbohydrate of the source leaves (L). Grass fructans are compared with standard oligoinulins from *Helianthus tuberosus*. Markers M, S N, I and K refer to the mobilities of standard monosaccharide, sucrose, neokestose, 1-kestose, and 6-kestose respectively. Numerals 4–12 mark the mobilities of oligoinulins of degree of polymerization 4–12. Sugars were visualized with urea-phosphoric acid stain. (Source: Cairns and Ashton, 1993).
fuel the process without the involvement of other cofactors. The integration of inulin synthesis and degradation is thought to be mediated by SST, which is detectable in tubers during synthesis, but not during remobilization. Because the FFT reaction is reversible, the poise of the reaction towards or away from chain elongation would depend on the input of carbon via SST, favoring depolymerization when its activity is absent. A third enzyme, fructan exo-hydrolase (FEH) was implicated in the mobilization of fructan, releasing free fructose for subsequent resynthesis into sucrose for export.

With the recent purification of SST and FFT enzymes from *H. tuberosus* tubers and chicory roots (Koops and Jonker, 1996; Luscher et al., 1996; van den Ende and van Laere, 1996) this model for synthesis has been largely confirmed with two modifications: a) The SST is not monofunctional, since it also forms oligoinulins of DP > 3 from sucrose. b) In vitro reconstitution of SST and FFT allows the synthesis of inulin up to DP ≤ 15 from sucrose. The original model required separate
compartmentation of FFT (vacuole) and sucrose plus SST (cytoplasm) because FFT-mediated chain elongation in vitro was markedly inhibited by sucrose (Edelman and Dickerson, 1966). The recent demonstrations of FFT activity in the presence of sucrose and SST activity obviates this necessity. The general model for fructan synthesis in Asparagus roots and onion bulbs is thought to be analogous to that of H. tuberosus, though a third enzyme, a \( \delta^6 \)-fructosyl transferase is implicated to explain the occurrence of the neokestose series of fructan in these species. The enzymological data for these species is not as full or as conclusive as the recent data for H. tuberosus and chicory (Cairns, 1992a, 1993).

**B. Fructan Synthesis in Leaves of Temperate Gramineae**

Experimental perturbation of carbon partitioning in grass leaves is straightforward and has provided an excellent system for the study of fructan metabolism and its enzymology and by contrast with root sink systems, the physiology of the grass leaf system is well characterized (Wagner et al., 1983; Housley and Pollock 1985; Cairns and Pollock, 1988a,b; Simpson et al., 1991; Cairns et al., 1997). Leaves manipulated to contain low levels of photoassimilates fully degrade sucrose and fructan, and loose both the capacity for in vivo fructan synthesis and extractable fructosyl transferase activity (Wagner et al., 1986; Cairns and Pollock, 1988a,b; Obenland et al., 1991; Cairns, 1992b). When such tissues are subsequently placed into positive carbon balance, sucrose accumulates until a threshold concentration of 15–20 mg g\(^{-1}\) fresh mass is reached, at which point tissue sucrose concentration stabilizes, conversion of sucrose into fructan is initiated and extractable fructosyl transferases are induced (the regulation of this process is discussed below). Synthesis of fructan occurs de novo, with sucrose as the sole precursor and with no requirement for (detectable) fructan primers. Fructan can accumulate at instantaneous rates similar to those of photosynthetic sucrose synthesis (e.g. 1.6 mg g\(^{-1}\) h\(^{-1}\) at 250 \( \mu E \) m\(^{-2}\) s\(^{-1}\)) and can reach tissue concentrations of 20–40 mg g\(^{-1}\) fresh mass. Fructan synthesis thus represents a massive component of primary carbon metabolism, contributing substantially to resource allocation within the plant.

Most early enzymological studies in grasses detected 1-kestose as the sole transferase product from sucrose. These data were interpreted as evidence for a monofunctional SST, and cited as evidence for the applicability of the SST/FFT scheme, though little evidence was presented for further polymerization by FFT or otherwise. By implication, the only route for carbon into grass fructan was through 1-kestose, consistent with the model of Edelman and Jefford (1968). However, the emerging enzymological evidence in grasses is equivocal in its support for this model since the isolated fructosyltransferase activities are generally multifunctional and/or also catalyze hydrolytic reactions. In addition, all the enzymes so far described have properties which are difficult to reconcile with the physiology of fructan synthesis in the source tissue. There are four areas in particular where behavior in vivo conflicts with in vitro observations. a) Kinetics: Fructosyl transferases from grasses (and other genera, e.g. Koops and Jonker, 1996; Van den Ende and Van Laere, 1996), universally require high substrate concentrations for function in vitro. Their kinetic response generally approximates to linearity in the range 50–500 mM sucrose and concentrations for half maximal activity are in the range 100–300 mM. b) Rates of synthesis: In the few instances where direct comparisons have been made, the in vitro sucrose fructosyl transferase activity of grass leaves engaged in fructan synthesis are adequate to support the rates of flux of fructose into fructan in vivo (Cairns and Pollock, 1988a,b; Cairns, 1992b). However, the sucrose concentrations required to cause physiologically significant rates of fructosyl transfer markedly exceed those thought to exist in the vacuoles of grass mesophyll cells, the currently accepted site of fructan synthesis (Wagner et al., 1983). c) Fidelity in reproducing representative natural structures: The products of fructosyl transferases assayed in vitro often differ in linkage structure, and especially in size, from the native complement of fructans in the source tissue and, d) Absence of large fructans: Polymerization of the large \( \beta 2,6 \) linked fructans characteristic of grass tissues has been difficult to demonstrate in vitro, despite the magnitude of the process in vivo.

Reports of purified fructosyl transferases from grasses which have appeared recently illustrate some of the difficulties described above: in barley, two enzymes have been isolated, both exhibiting a requirement for high sucrose concentration and neither fully saturating at 600 mM. The first, described as 1-SST, synthesizes 1-kestose as the predominant product from sucrose, though higher oligosaccharides
of DP ≤ 5 were also formed (Simmen et al., 1993). Consistent with the recently reported data for SST from Helianthus and chicory, barley 1 -SST is at least bifunctional, having fructan-sucrase or FFT-like activity in addition to SST activity. Therefore, the current terminology is not wholly consistent with the range of activities exhibited by this enzyme in many different plant species. The second barley enzyme, termed 6-SFT (formerly 6-SST; Simmen et al., 1993; Duchateau et al., 1995), is also a multifunctional enzyme which, in the absence of 1-kestose, synthesizes 6-kestose directly from sucrose, providing an alternative route for carbon into fructan. 6-SFT also transfers single fructosyl residues from sucrose to carbon 6 of fructan residues in oligosaccharides of the inulin series, generating the branched fructans which occur naturally in barley leaves. Independently, the partial reactions of 1 -SST and 6-SFT can explain the synthesis of barley oligofructans up to ca.DP5, but there is no evidence either that the rates are physiologically significant or that reconstitution of the two activities can generate oligosaccharides de novo. In addition, there is no direct evidence that they are involved in synthesis of the larger fructans which also occur naturally in barley leaves. The third activity of the 6-SFT preparation is as a β-fructofuranosidase (invertase). With 200 mM sucrose as sole substrate, invertase accounts for 78% of the total activity. In contrast to its low sucrose-affinity for trisaccharide synthesis (Km > 300 mM), the value for sucrose hydrolysis is ca. 10 mM (Simmen et al., 1993). The detailed physiological consequences of this high-affinity hydrolytic activity, particularly in the early stages of de novo synthesis, remain to be explored. The properties of barley 1-SST and 6-SFT are clearly at variance with the SST/FFT model, suggesting as they do, potential roles in grasses for a) multifunctional transferases, b) direct sucrase-type fructosyl transfer, and c) an alternative route for fructosyl residues into fructan, via 6-kestose.

An enzyme analogous to the FFT activity from H. tuberosus, which catalyses non-synthetic transfer between pre-existing primer inulins to generate oligosaccharides of DP ≤ 6, has been isolated recently from Lolium rigidum (St. John et al., 1997a). This enzyme did not saturate with increasing substrate concentration, even at 400 mM 1-kestose. These authors note that this species accumulates predominantly high Mr fructan with β2,6-linkages. The predominant affinity of the isolated FFT, by contrast, was for low Mr, β2,1-linked fructans. It showed no detectable donor or acceptor activity against β2,6 linkages and could not, therefore, account for their synthesis in the source tissue. When this FFT was incubated simultaneously with 100 mM sucrose and a partially purified, multifunctional preparation having SST, FFT and 6-kestose-forming activity, oligosaccharides of DP ≤ 6 were synthesized (St. John et al., 1997b). The only circumstances under which traces of larger fructans were generated were in experiments where sucrose was periodically added to the assay to maintain a concentration of 100 mM over a 10h reaction. These larger products contained both 2,6 and 2,1 linkages, which is interesting in the view of the absolute β2,1 specificity of the FFT when assayed alone and the β2,6 specificity of high Mr fructan synthesis in vivo. Clearly there is no simple relationship between the enzymological and physiological data. The evidence from L. rigidum supports the Edelman and Jefford model to the extent that a fraction active against sucrose is separable from an FFT active against fructan only. However, because the structure of the FFT product differs from the native fructan and because the kinetic parameters and rates of product formation have not been compared with the source tissue, the physiological significance of these observations is not fully resolved.

Since, for the reasons outlined above, it is often difficult to place incomplete enzymological data into a physiological context, we regard the demonstration of in vitro polymerization of authentic grass fructan at physiological rates as a prerequisite for the understanding of the process in vivo. We have shown that this can be achieved using a combination of high concentrations of partially purified enzyme and high sucrose concentration (Cairns, 1992b). The chromatographic fingerprints of the products are species-specific and strongly resemble the native fructan complement in the tissue used as the source of the enzyme (compare the species in Fig. 2). In the case of Phleum pratense we have established, using a combination of anion exchange chromatography-PAD and methylation/GC-MS analysis, the enzymatic synthesis of 2,6-linked polymers of high Mr (up to DP = ca. 50, Fig. 3). Because, a) large fructans representative of the native polymers are synthesized de novo from sucrose at physiological rates, b) the products are species-specific, and c) the pattern of sucrose-regulation and its sensitivity to inhibitors of gene expression parallels that of the tissue (Cairns, 1992b; Cairns and Ashton 1993), we can be reasonably certain that the in vitro activities are
involved in fructan synthesis in vivo.

The enzyme concentration required for this high fidelity synthesis in vitro, (>ca. 5.0 g fr mass equivalent cm⁻¹; 10–14 nkat cm⁻¹), is at least 10-fold greater than that generally employed in enzymological studies of fructan synthesis. The apparent Km for polymerization in L. temulentum and P. pratense is in the range 200–300 mM sucrose, though the reactions typically tend to saturation only at above 1.0 M sucrose. To emulate in vivo rates of accumulation of polymeric fructan, the substrate concentrations required for enzymatic synthesis are in the range 1.0–1.5M. The pH optima for these polymerising reactions are 5.5–6.0 (Cairns and Ashton, 1994) and the polymerising fractions have Mr of 50–60 kD based on size exclusion chromatography (Cairns et al., 1997, 1999). Km, Mr and pH optima for polymerization are similar to those generally reported for trisaccharide and oligosaccharide formation by fructosyl transferases (SST, SFT and FFT) from most plants.

It is clear that fructosyl transferases assayed at a range of enzyme concentrations exhibit different product specificities, higher enzyme concentrations producing larger fructans over the same incubation period and with otherwise identical conditions of reaction (Cairns, 1995). Given the strong similarity in general properties between extracts which produce large fructans and ones which will only synthesize oligosaccharides, it is possible to explain the absence of polymerization by the latter, in terms of a requirement for high enzyme and substrate concentration. Enzymes assayed at low concentration and reported as ‘SST’ and ‘6-SFT’ may well polymerize larger fructans under different reaction conditions. The oligosaccharide synthesis (DP > 3) observed for Helianthus and chicory SST may also be enhanced at higher enzyme concentration.

All reported enzymes of fructan synthesis exhibit peculiarities in specificity and/or kinetics which need to be considered against the conditions in vivo. Our polymerization reactions are no exception. Whilst they make realistic fructans at realistic rates and the pattern of regulation parallels that in vivo, the physiological consequences of the in vitro properties of the polymerase demand closer examination. From the known in vivo and in vitro rates of synthesis in leaves of 1.4 nkat g⁻¹ (Cairns and Pollock, 1988b; Cairns and Ashton, 1994), and estimates of mesophyll vacuolar volume at 441 mm³ g⁻¹ fresh mass (Cairns et al., 1989), the concentration of synthetic activity in vivo could theoretically reach 3.2 nkat cm⁻³, less than one third the concentration needed in vitro to sustain this rate of synthesis. But to achieve even this activity would require a substrate concentration of more than 1.0M (Cairns and Ashton, 1994). By mensuration (Cairns et al., 1989) and by direct measurement, (Koroleva et al., 1997) the vacuolar sap of fructan-accumulating mesophyll cells contain sucrose at 100–200 mM, which would result in an activity of only 10–15% of the necessary rates. If the properties of in vitro polymerization reflect the situation in vivo, there is both insufficient enzyme and insufficient substrate in the vacuole to explain the rates of accumulation within the tissue. Kaeser (1983) reported micro-vesicular localization of inulin synthesis in tubers of H. tuberosus. It remains a possibility that high substrate and enzyme concentration could co-exist in such vesicles in grasses, satisfying the requirements for enzymatic polymerization in vivo. This can be reconciled with findings localizing fructans and their enzymes in isolated vacuoles (Wagner et al., 1983; Frehner et al., 1984; Cairns et al., 1989) since presumably such vesicles would fuse with the tonoplast resulting in a final location of both dilute enzyme and product in the vacuole.

C. Fructan Metabolism in Other Tissues of Temperate Gramineae

In terms of mass, the developing stem of cereal and grass inflorescence accumulates more fructan than any other organ (Pollock and Jones, 1979). Although the upper internode and the peduncle do contain chlorophyll, the majority of the carbon stored in the stems comes from leaf photosynthesis (Austin et al., 1977). Photosynthetic from lower leaves appears more likely to accumulate as fructan in stems, contrasting with photosynthetic from flag leaves, most of which passes directly into the ear (Pearman et al., 1978). Fructan concentrations can reach up to 40% of dry weight around the time of anthesis.

In annual cereals, up to 50% of stem dry weight is lost during grain maturation (Bonnett and Incoll 1992) and fructan contents decline markedly. The proportion of mobilized fructan which promotes grain filling is not easy to determine and may depend upon both genotype and cultivation conditions (Schnyder 1993). There are also indications that mobilization of stem fructan is induced developmentally, rather than in direct response to increased
demand for assimilate by the ears. (Kühbauch and Thome, 1989; Bonnett and Incoll, 1992). There have been few biochemical studies on stem fructan metabolism because of the recalcitrance of the tissue. Dubois et al., (1990) showed that SST activity in spring wheat stems showed genotypic differences which correlated both with initial sucrose content and with the rate of subsequent fructan accumulation. Elevated CO₂ concentrations increased sucrose contents, extractable SST activity and final stem fructan content. This experiment was carried out under controlled environment conditions where total soluble carbohydrates accumulated throughout the experiment. Under field conditions (Bancal and Triboi, 1993) there was little correlation between SST activity and fructan content. These authors suggest that net accumulation was regulated by changes in FEH activity which were associated with mobilization of stored fructan during grain filling. Changes in FEH activity are also associated with mobilization of fructan from stems of Dactylis glomerata (Yamamoto and Mino, 1989). In these cases the assumption is that activity changes would be caused by changes in the amount of enzyme protein and are under developmental control as well as being sensitive to assimilate abundance.

The development by Silk (1984) of the continuity equation allowed measurements of composition and elemental growth rate to be used to calculate assimilate fluxes into meristematic and extending tissues undergoing ‘linear’ growth. This has led to the demonstration of a substantial involvement of fructan metabolism during the growth of grass and cereal leaves. The main contribution to studies of this kind has been by Schnyder, Nelson and co-workers (Schnyder, 1986; Schnyder et al., 1988; Schnyder and Nelson, 1987, 1989). They demonstrated very large fluxes of material into fructan within the extension zone. As the segment of tissue aged, and moved further up the developing leaf, there was an equivalent flux out of fructan and into structural materials. Our own studies have shown that these fluxes are sensitive to changes in growth rate (associated with chilling) and assimilate abundance (caused by increased photoperiod or elevated CO₂), but that fructans remain the major temporary sink within the extension zone. We have also demonstrated that elevated CO₂ strongly stimulates hexose accumulation and that increased carbohydrate contents are associated with increased rates of respiration, regardless of growth rate (Table 3).

Because of the extremely stable gradient of tissue development along the leaf, this experimental system has considerable potential for the study of metabolic regulation. To date, enzymological studies have been constrained by the small mass of tissue involved.

Developing cereal grains also accumulate fructan. Final concentrations only reach 1–2% of the dry weight (MacLeod and McCorquodale, 1958) but the proportion in young developing grain is much higher (Escalada and Moss, 1976). Measurements of SST and FFT activities (Housley and Daughtry, 1987) suggest that active fructan accumulation occurs very early in grain development, but that the fructan pool becomes progressively less accessible. During the main phase of starch biosynthesis, fructan forms a static pool making up a progressively declining proportion of total grain carbohydrate. It is not known either how this process is regulated or what its physiological significance is, although it has been suggested that synthesis of sucrose into fructan would facilitate continued passive unloading from the phloem (Hendrix, 1983).

### Table 3. Respiration rate in extension zones of cereal leaves as related to growth treatment and soluble carbohydrate content

<table>
<thead>
<tr>
<th>Growth treatment</th>
<th>Respiration rate (nmol O₂ mg⁻¹ h⁻¹)</th>
<th>Carbohydrate content (mg g⁻¹ fresh wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 °C:350ppm CO₂</td>
<td>18</td>
<td>10.2</td>
</tr>
<tr>
<td>20 °C:500ppm CO₂</td>
<td>125</td>
<td>18.6</td>
</tr>
<tr>
<td>5 °C:350ppm CO₂</td>
<td>163</td>
<td>20.3</td>
</tr>
</tbody>
</table>


D. Fructan Hydrolysis in Leaves of Temperate Gramineae

In general, studies of fructan hydrolysis have concentrated on non-photosynthetic tissues such as storage organs, cereal stem interposed and the stubble remaining after defoliation. Recent reviews have summarized such studies (Housley and Pollock, 1993; Simpson and Bonnett, 1993). This section summarizes our current understanding of fructan hydrolysis, emphasizing more recent reports.

Fructan mobilization may be under developmental control (as in the case of inulin breakdown in sprouting tubers of H. tuberosus; Edelman and Jefford, 1968), but in many cases it appears to be sensitive to the balance between supply of and demand for fixed carbon. It is not clear how the process is regulated to prevent net degradation of fructan during periods of
assimilate surplus. Leaves manipulated to be in negative carbon balance can completely hydrolyze any fructan and sucrose present and export the carbohydrate to sinks (Cairns and Pollock, 1988a; Simpson et al., 1991). In general, sucrose and smaller fructans are degraded very quickly whilst higher Mr fructans tend to be more persistent. There is good evidence for exo-hydrolysis of single terminal fructose being the major pathway of breakdown (Pollock, 1982). The hexose products, fructose and a small amount of glucose (terminal sucrose will be hydrolyzed by invertase), are assumed to be transferred from the vacuolar site of hydrolysis to the cytoplasm where they are phosphorylated via hexokinase and resynthesized into sucrose.

Fructan hydrolase activities isolated from plants are universally exo-hydrolytic and are termed fructan exohydrolase (FEH). Thus, hydrolysis of fructan in vitro parallels the process in vivo. Exohydrolases from grasses exhibit pH optima in the range 4.5–5.5 and have Mr of 40–69 kD (Simpson and Bonnett, 1993). There is emerging evidence that ‘FEH’ is not one enzyme but a number of isoforms with differing molecular properties and specificities (Bonnett and Simpson, 1995). In common with synthesis, the enzymology of fructan hydrolysis has been difficult to reconcile with the known physiology of fructan mobilization. The main areas of discrepancy are: a) Kinetic: High substrate concentrations are required for in vitro function of FEH. Reports of Km vary widely between 0.22 and 89% w/v fructan but are generally c. 20% w/v (Simpson and Bonnett, 1993). Given that maximal concentrations of fructan in leaf tissue are around 4% w/v, these enzymes are likely to function at well below maximal rates in vivo. In addition, the rate of activity will be sensitive to substrate concentration and will be further limited as hydrolysis proceeds, particularly since many of the preparations show inhibition by free fructose. b) Enzyme specificity: The majority of grass hydrolases are assayed with, and preferentially hydrolyze β2,1 fructan, which is not the form predominantly accumulated in the source tissue. There have, however, been recent reports of activities with a preference for β2,6 bonds (Bonnet and Simpson, 1995; Henson and Livingston, 1996; Marx et al., 1997). c) Timing: Unlike the inducible enzymes of synthesis, many tissues contain ‘constitutive’ FEH activity, regardless of carbohydrate status. During fructan synthesis in grass leaves, for example, there is enough extractable FEH activity to counteract observed rates of fructan accumulation, particularly as small oligofructans produced early in accumulation are especially susceptible to attack (Simpson et al., 1991; Cairns et al., 1997). Because synthesis, accumulation and hydrolysis of fructan are all currently thought to be vacuolar, it is difficult to see how intermediate oligofructans persist for long enough to permit polymer building unless there is some form of compartmentation or in vivo inhibition of hydrolysis occurs. d) Regulatory: During fructan mobilization, extractable FEH activity often increases. However, the increase occurs after substantial fructan breakdown has already taken place (Simpson et al., 1991). One explanation for this may be that, because of the low affinity of FEH for the substrate, an increase in the absolute amount of enzyme is required to support hydrolysis at low substrate concentrations (Simpson and Bonnet, 1993).

In overview, studies of FEH are practically difficult and interpretation of the results complicated. Despite this, progress has been made with the recent purification of hydrolases with appropriate specificities for native grass fructan (Bonnet and Simpson, 1995; Henson and Livingston, 1996; Marx et al., 1997). However, the complexities of the process will make it difficult to unambiguously assign function in vivo until specific activities can be abolished either by mutation or by antisense technology.

### IV. The Control of Fructan Metabolism

#### A. Induction by Sucrose Accumulation

The clearest picture of the regulation of fructan synthesis is provided by the well characterized grass leaf system. When grown at low irradiance under a short photoperiod, leaves will not accumulate fructans. Excision and continuous illumination leads, as indicated above, to an increase in the concentration of sucrose and to the progressive appearance of fructans of increasing size. Treatment of leaves at the time of excision with inhibitors of gene expression blocks the conversion of sucrose into fructan without altering the total amount of soluble carbohydrate accumulated in the tissue (Wagner and Wiemken, 1987; Cairns and Pollock, 1988b; Table 4). Application of inhibitors at different times after excision indicated that the ability to convert sucrose into fructan was acquired fully within six hours of excision and illumination. After this, applications of
As indicated above, the convenience of the excised leaf system has meant that almost all detailed studies on the biochemistry, enzymology and molecular biology of fructan metabolism in Gramineae has been carried out using leaves. Studies on other tissues in grasses and cereals and on storage organs in the Liliales and the Asterales have not, however, suggested that any radically different mechanisms are operating elsewhere, so the current assumption is that the regulatory factors operating in leaves are probably of general significance.

cycloheximide or cordycepin had no effect (Winters et al., 1994). These observations led to two significant conclusions. The first was that regulation of fructan metabolism occurs at the level of coarse control, i.e. changes in the absolute amount of the enzyme(s) which synthesize fructan or, perhaps less likely, the amount of a strong activator of existing enzyme. The second is that sucrose concentrations per se do not appear to affect carbon fixation in the short term. The rate of carbohydrate accumulation is constant over the first 54 h of leaf excision (Housley and Pollock, 1985) and is insensitive to inhibitors which block the conversion of sucrose to fructan (Cairns and Pollock, 1988b). By this stage, soluble sugars can make up 40% of the dry weight of the leaf! Feeding exogenous sugar in the dark to excised leaves also leads to a similar induction of fructan biosynthesis (Wagner and Wiemken, 1987; Table 5), leading to the conclusion that it is the rise in sucrose or in some clearly related metabolite, which triggers the induction of fructan biosynthesis. (Wiemken et al., 1995).

B. Coarse Control by Altered Gene Expression

As indicated above, the convenience of the excised leaf system has meant that almost all detailed studies on the biochemistry, enzymology and molecular biology of fructan metabolism in Gramineae has been carried out using leaves. Studies on other tissues in grasses and cereals and on storage organs in the Liliales and the Asterales have not, however, suggested that any radically different mechanisms are operating elsewhere, so the current assumption is that the regulatory factors operating in leaves are probably of general significance.

Table 4. Quantitative analysis of water-soluble carbohydrate fractions from excised leaves illuminated in the presence of inhibitors of gene expression.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>Total water-soluble carbohydrate accumulated in 24 h (mg g⁻¹ fresh mass)</th>
<th>Proportion with DP &gt;2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water control</td>
<td>–</td>
<td>43.3</td>
<td>58.4</td>
</tr>
<tr>
<td>L-MDMP</td>
<td>10</td>
<td>44.5</td>
<td>53.0</td>
</tr>
<tr>
<td>D-MDMP</td>
<td>10</td>
<td>40.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>100</td>
<td>40.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>1000</td>
<td>44.9</td>
<td>2.8</td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>1000</td>
<td>48.6</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Excised leaves of Lolium temulentum were stored in aqueous solutions of metabolic inhibitors for the initial 3 h of a 24-h illumination period. At the end of this period tissue was extracted and water-soluble carbohydrate analysed by high-performance liquid chromatography. Source: Cairns and Pollock, 1988b.

Newly synthesized proteins can be monitored very effectively in excised leaves by administering 35S methionine to the cut ends. Changes in mRNA can also be monitored by cell-free translation. In

Table 5. The effects of exogenous sugars on fructan biosynthesis and fructosyl transferase activity in detached barley leaves kept in the dark.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Fructosyl transferase activity (nkat ml⁻¹)</th>
<th>Fructan content (mg g⁻¹ fresh mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.64</td>
<td>1.48</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.99</td>
<td>2.07</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>0.50</td>
<td>0.38</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0.48</td>
<td>0.01</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.47</td>
<td>1.08</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.22</td>
<td>0.31</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.33</td>
<td>0.43</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.31</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Sugars were supplied for 16 h by standing leaf blades in 0.5M solutions. Data from Wagner, Wiemken and Matile, 1986.

The regulation of fructan metabolism is intimately connected with the various roles of sucrose within the overall syndrome. Sucrose can act as both a fructosyl donor and as a fructosyl acceptor. There is also evidence that sucrose prevents the inactivation of fructan-synthetic enzymes (Obenland et al., 1991; Cairns and Ashton, 1994), and protects small fructan intermediates from the action of hydrolases during polymer building (Cairns et al., 1997). In many systems sucrose is known to modulate carbon metabolism via fine control of existing enzyme activities and via its influence on the patterns of gene expression. It seems probable that all of the factors integrate to regulate the flow of carbon into fructans within leaves.

As indicated above, the convenience of the excised leaf system has meant that almost all detailed studies on the biochemistry, enzymology and molecular biology of fructan metabolism in Gramineae has been carried out using leaves. Studies on other tissues in grasses and cereals and on storage organs in the Liliales and the Asterales have not, however, suggested that any radically different mechanisms are operating elsewhere, so the current assumption is that the regulatory factors operating in leaves are probably of general significance.
both cases, excised leaves fed sucrose in the dark showed a relatively small number of novel polypeptides which were synthesized up to about 8 h after excision, suggesting that only a few genes were involved. Illuminated leaves showed larger changes in both protein synthesis and cell-free translation, suggesting that photosynthetic processes were affected even though this did not result in declining assimilation rates (Winters et al., 1994). Differential screening of cDNA libraries from induced leaves using probes derived from induced and uninduced leaves revealed a number of clones exhibiting increased expression. One of these was analyzed further and showed extremely strong sensitivity to both increases and decreases in sucrose concentration. This gene was also more highly expressed in lines of *Lolium perenne* which accumulate higher amounts of soluble carbohydrates (L. Skot, personal communication). Unfortunately, sequence analysis indicated that the DNA sequence coded for S-adenosyl methionine synthetase rather than for an enzyme capable of using sucrose as a substrate (Winters et al., 1995). Subsequently, however, heterologous screening of a similar library with probes derived from maize root invertases has identified a gene sequence with close homology to the invertase family and to the barley SFT (Sprenger et al., 1995). This gene is strongly up-regulated in the presence of sucrose (J. Gallagher and C. Pollock, unpublished). This suggests that there are genes in fructan accumulating tissues which are up-regulated in the presence of sucrose and which code for enzymes which metabolize that sucrose, potentially into fructan. The up-regulated clone from *L. temulentum* produces a protein which cleaves sucrose but which will also catalyze the synthesis of higher oligosaccharides (J. Gallagher and C. Pollock, unpublished). The clone isolated and sequenced by Sprenger et al. (1995) also codes for an enzyme which is capable of both hydrolysis and fructosyl transfer and which is more closely related to higher plant invertases than to bacterial fructosyl transferases. These authors argue that the fructan syndrome in plants has arisen polyphyletically through a range of modifications of various members of the acid β-fructofuranosidase (invertase) family (Wiemken et al., 1995). Presumably such modifications would have been of selective advantage when they occurred in genes which were up-regulated by high levels of assimilate.

There is less direct evidence that the enzymes of fructan hydrolysis are regulated in a similar manner. Possible increases in assimilate abundance and fructan accumulation are associated with reductions in extractable fructan exohydrolase activity (Simpson et al., 1991) and in some cases these changes are sensitive to inhibitors of gene expression (Wagner et al., 1986). It is not known whether specific gene-products coding for enzymes which degrade fructan are down-regulated under such conditions, but the increasing availability of clones for enzymes of fructan synthesis and the likelihood that fructan hydrolases will also show sequence homology to invertases should lead to the development of specific probes to measure changes in message abundance, and of specific antibodies to estimate enzyme protein levels.

**C. Fine Control via Metabolites in Photosynthetic Tissue**

As indicated above, the rate of sugar accumulation in excised, illuminated grass leaves remains constant until chlorophyll degradation sets in (Housley and Pollock, 1985). The rates are similar in magnitude to the maximum rate of leaf photosynthesis measured by gas exchange (Natr, 1969). If one assumes that sucrose concentrations are similar in cytosol and vacuole and that the maximum sucrose content stabilizes at around 20 mg fresh mass, this would equate to a uniform concentration change from zero to ca 60–100 mM during the course of the experiment. Any selectivity which would lead to high concentrations of sucrose in the cytosol (as suggested by Winter et al., 1993) would, of course, amplify the effect. In plants where chloroplast starch forms the major leaf carbohydrate reserve, much smaller increases in sucrose concentration are thought to feed back, via elevated concentrations of the regulating fructose 2,6 bisphosphate, to slow export of triose phosphate from the chloroplast and stimulate starch biosynthesis by the activation of ADPG pyrophosphorylase (Stitt, 1996). This feedback does not apparently occur in grass leaves when sucrose accumulation was stimulated by chilling the sink tissue (Table 6). We investigated the possibility that the cytoplasmic fructose 1,6 bisphosphatase for *L. temulentum* was less sensitive to fructose 2,6 bisphosphatase, but this was not the case (Collis and Pollock, 1991), suggesting that the inhibition observed in vitro is over-ridden in vivo, possibly via elevated triose phosphate concentrations. This ‘isolation’ of
chloroplast metabolism from large changes in cytosolic sucrose concentrations appears to be a significant element of the fructan syndrome in Gramineae and may be part of its selective advantage. Many grasses evolved in an environment where, through shading; a perennial growth habit or herbivory, there would be large and rapid changes in the balance between supply of and demand for fixed carbon. These would result in large changes in the fluxes through the competing pathways of primary carbon metabolism.

There is a further aspect of fine control of fructan metabolism which must be distinctive, although the evidence for its occurrence is circumstantial. The stoichiometry of fructan biosynthesis (via the accepted model of direct fructosyl transfer from sucrose) liberates one mole of glucose for every mole of fructose which is transferred. However, radiotracer experiments demonstrate unequivocally that almost all the radioactivity present in sucrose after feeding $^{14}$CO$_2$ is eventually accumulated in fructan (Pollock, 1979). Recycling of glucose must, therefore, occur, presumably via hexokinase and sucrose phosphate synthase. Such a recycling pathway would have two effects. Firstly, elevated flux through hexokinase could invoke the signaling responses associated with down-regulation of gene expression (Jang and Sheen, 1996) and secondly, the flux through sucrose phosphate synthase would increase dramatically in relation to that through cytoplasmic fructose 1,6 bisphosphatase (Collis and Pollock, 1992). Direct measurements of enhanced flux or of the activation state of sucrose phosphate synthase (Huber et al., 1995) have not yet been made under these conditions. There have been suggestions that there may be direct transfer of fructose residues from UDP fructose to the growing fructan chain (Pontis, 1995). If this suggestion is substantiated, then it would reduce the flux through the pathways discussed above.

**D. Compartmentation of Sucrose and Fructan Metabolism**

### 1. Intracellular Compartmentation

Sachs (1894) was the first researcher to propose a role for the vacuole in the storage of carbohydrates. By using ethanol to precipitate fructans, he observed the resulting sphaerocrystals in the vacuoles of members of the Asterales. For many years after that, only indirect evidence was available to support the hypothesis, but the ability to prepare isolated vacuoles permitted a direct examination of distribution (Pollock and Kingston-Smith, 1997). Measurements on enzymatically-released protoplasts and vacuoles indicate unequivocally that both the putative enzymes of fructan metabolism and the substrates and products can be found in vacuoles (Table 7). Concerns still exist over the disparity between the catalytic constants of putative enzymes measured in vitro and the apparent sucrose concentrations within vacuoles, but there seems little doubt that the vacuole is the major site of storage (Pollock and Kingston-Smith, 1997). It has been proposed, however, that fructan synthesis (as opposed to storage) may occur in small vesicles which subsequently fuse with the vacuole (Kaeser, 1983). Final resolution of these disparities will depend upon the purification of all the enzymes involved and the use of specific antibodies to localize these within the cell.

### 2. Intercellular Compartmentation

Jellings and Leech (1982) estimated that photosynthetic mesophyll cells make up only 55% of the cell population in cereal leaves, whereas they would make up in excess of 90% of the cells used to prepare isolated vacuoles in the experiments described above. Histochemical analysis of starch in barley leaves has already demonstrated discontinuities in the distribution between mesophyll cells and the photosynthetic cells of the parenchymatous bundle sheath (Williams et al., 1989) and there is no a priori reason for discounting such discontinuities in the metabolism of sucrose. Histochemical localization of acid invertase has demonstrated high concentrations near the vasculature. (Kingston-Smith and Pollock, 1996).
Using the techniques of single-cell sampling (Tomos et al., 1992) increases in fructan metabolism in mesophyll and bundle sheath have been shown to be clearly linked, with vacuolar invertase activity almost undetectable, leading to the conclusion that sucrose hydrolysis in leaves of temperate Gramineae is spatially separated from fructan biosynthesis (Koroleva et al., 1997). Once again, histochemical location of relevant proteins and mRNA species will help to resolve the magnitude of gradients in primary carbon metabolism within leaves, but any tissue-level compartmentation has very significant consequences for models of regulation based upon measurements of whole tissue extracts.

V. Fructan Biosynthesis in Transgenic Plants: A Physiological Perspective

Progress with the identification, purification, sequencing and cloning of plant enzymes with fructosyltransferase activity has been achieved only in the last two years, presumably due to the equivocal and ambiguous nature of much of the earlier enzymological data (Pollock and Cairns, 1991: Cairns, 1993). Hence, work with transgenics has largely employed the available, well characterized levansucrases from bacteria, mainly the Sac B gene from Bacillus spp and levansucrase from Erwinia amylovora (Röber et al., 1996). These levansucrases synthesize very large fructan (Mr. 10^5–10^6D, termed levan) from sucrose as sole precursor. Stable transformation of fructan non-accumulators such as potato, maize and tobacco has been reported and significant accumulation of levan demonstrated, ranging from 2–19% of tissue mass (Table 8). The stated rationales for the production of such plants are: a) Experimental: to produce plants with altered source-sink balance for the experimental investigation of carbon partitioning (Röber et al., 1996). b) Industrial: to provide sources of fructan qualitatively and quantitatively improved by comparison with that from ‘natural’ sources (Ebskamp et al., 1994). c) Aimed at crop improvement: to improve drought tolerance, since enhanced fructan content is believed by some to mediate stress tolerance in endogenous accumulators (Pilon-Smits et al., 1995a,b).

The Sac B transformants provide a number of interesting enigmas for the physiologist. The fructan accumulated in Sac B transformants is cited as evidence for major alterations in reserve carbon partitioning. While the transformants clearly accumulate large amounts of levan of high Mr, we question the significance of this accumulation in terms of alterations to instantaneous carbon flux and partitioning; The fructan concentrations are cited as end-point values, without consideration of the accumulation period or the accumulation rate. Without such observations, comparisons with the magnitude of endogenous carbon fluxes cannot be made. From the few studies which do cite accumulation period, net rates of formation can be estimated and compared with rates of fructan synthesis in the leaves of an endogenous accumulator (L. temulentum) or with starch accumulation in sinks of untransformed starch accumulators (developing pea and maize seed). Such an analysis is summarized in Table 8.

The transformants accumulate fructan at rates which are markedly lower than those observed in the natural fructan accumulator. The expressed rates of net levansucrase activity are correspondingly small when compared to the magnitude of the fluxes through endogenous pathways of primary carbon metabolism in untransformed starch accumulators, as indicated by natural starch accumulation rates. These metabolic comparisons show that the proportion of current photosynthate flux directed into levan is very small in the Sac B transformants. The accumulation is, therefore, a result of minor leakage of carbon into fructan over long periods into a chemical compartment which cannot be remobilized by the plant.
The apparent alterations in reserve metabolism are also of interest in the context of the conditions of plant culture used. Some transformants have been grown at irradiances reported at 3000 lux (van der Meer et al., 1994), equivalent to less than 40 \( \mu \text{E m}^{-2} \text{s}^{-1} \) PAR: (converted according to McCree, 1972) and 42 \( \mu \text{E m}^{-2} \text{s}^{-1} \) (Pilon-Smits et al., 1995a). These values are low and close to the light compensation point for C3 plants (Milthorpe and Moorby, 1974). At these irradiance values it is, perhaps, surprising that the plants were even in positive carbon balance and involved in reserve synthesis. The rationale for the use of low irradiance was not reported. However, Sac B transformants of tobacco produced deleterious necrotic lesions associated with high carbohydrate status when grown at higher irradiance. (A. Snow and R. J. Simpson, personal communication). They may explain the choice of lower PPFD in the other experiments.

The transformants are consistently reported as not containing Sac B mRNA detectable by hybridization, the enzyme protein cannot be detected immuno-

<table>
<thead>
<tr>
<th>Species/Tissue/transformant/treatment</th>
<th>Polysaccharide</th>
<th>Accumulated polymer (mg g(^{-1}) fr. mass)</th>
<th>Accumulation period (d)</th>
<th>Accumulation rate(^1) (nkat g(^{-1}) fresh mass)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize, kernel cytoplasm</td>
<td>Levan (Sac B)</td>
<td>7.5</td>
<td>55</td>
<td>0.010</td>
<td>Caimi et al., 1996</td>
</tr>
<tr>
<td>Maize, kernel vacuole</td>
<td>Levan (Sac B)</td>
<td>18</td>
<td>30</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>Potato leaves: KP7</td>
<td>Levan (Sac B)</td>
<td>4.5</td>
<td>49</td>
<td>0.007</td>
<td>Pilon-Smits et al., 1996</td>
</tr>
<tr>
<td>Potato leaves: KP17</td>
<td>Levan (Sac B)</td>
<td>4.7</td>
<td>49</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Tobacco KP; drought control</td>
<td>Levan (Sac B)</td>
<td>0.02</td>
<td>11</td>
<td>0.0001</td>
<td>Pilon-Smits et al., 1995a</td>
</tr>
<tr>
<td>Tobacco KP -0.4kPa</td>
<td>Levan (Sac B)</td>
<td>0.05</td>
<td>8</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>Tobacco KP -0.8kPa</td>
<td>Levan (Sac B)</td>
<td>0.275</td>
<td>11</td>
<td>0.0018</td>
<td></td>
</tr>
<tr>
<td><em>L. tulipulentum</em> leaf</td>
<td>Fructan (endogenous)</td>
<td>20</td>
<td>1</td>
<td>1.4</td>
<td>Cairns and Pollock, 1988a</td>
</tr>
<tr>
<td>Maize, kernel</td>
<td>Starch (endogenous)</td>
<td>319</td>
<td>28</td>
<td>0.8</td>
<td>Ozburn et al., 1973</td>
</tr>
<tr>
<td>Pea seed</td>
<td>Starch (endogenous)</td>
<td>167</td>
<td>18</td>
<td>0.7</td>
<td>Milthorpe and Moorby 1974</td>
</tr>
</tbody>
</table>

\(^1\) Calculated as anhydrohexose \( M_r = 162 \).

0logically and the enzyme activities are at the limit of detection (Ebskamp et al., 1994; van der Meer et al., 1994; Pilon Smits et al., 1995). Sensitive \(^{14}\)C enzyme assays are required to detect levan formation in vitro (Ebskamp et al., 1994; van der Meer et al., 1994; Röber et al., 1996), whereas, for natural accumulators, fructan production in vitro is readily detectable without the use of radioisotope (e.g. in Figs. 2 and 3). These observations are in apparent contradiction to the large tissue concentrations of levan reported, but can be rationalized in the context of the low expression of levansucrase in the transformants demonstrated in Table 8.

The properties of the levansucrase enzymes, in the context of the physiology of the plant, have also been neglected in discussion of the transformed plants. For the bacterial enzymes, the \( K_m \) for sucrose is between 20–60 mM (Han, 1990) and is 20 mM for the Sac B gene product (Dedonder, 1966). This is of particular physiological interest. In only a few instances have transformant tissue sucrose concentration and dry weight:fresh weight ratio been reported.
(Ebskamp et al., 1994; Pilon Smits et al., 1995). This allows estimation of mean tissue, and hence vacuolar, sucrose concentration. The value in each case was less than 3 mM so the enzyme would be functioning at less than 7% of \( V_{\text{max}} \). This would, of course, contribute to the very low rates of expression observed and make accumulation rate extremely sensitive to changes in tissue sucrose concentration.

In relation to drought stress, Sac B transformants of tobacco contained increased levan concentration when subjected to PEG-induced osmotic stress (Pilon-Smits et al., 1995a). Fructan accumulation was correlated with improved drought tolerance (as measured by dry weight increment), despite being under the control of the constitutive (i.e. stress-insensitive) e35S CaMV promoter (Ebskamp et al., 1994). It is interesting that sucrose accumulation in the stressed transformants also increased by 13-fold relative to the (-)drought control. This corresponds to an estimated change of 0.2–2.8 mM sucrose in the tissue sap. The resulting theoretical substrate-dependent stimulation of the Sac B enzyme of ca. 16-fold could alone explain the difference in levan content between control and droughted plants. This leaves open the question of whether the increase in levan accumulation was a direct or indirect consequence of the stress treatment, a debate which is equally valid for natural fructan accumulators.

In summary, the data from the bacterial transformants provide an exciting and interesting new departure for studies of assimilate partitioning, though some of the conclusions benefit from closer re-examination from a physiological perspective.

VI. Concluding Remarks

Natural fructan accumulators are unique in the sense that they possess a mechanism for reserve carbon allocation which differs grossly in its enzymology, compartmentation and regulation, from 90% of higher plant species. Endogenous fructan metabolism represents a distinctive extension of sucrose metabolism, an understanding of which also serves to illuminate factors governing primary carbohydrate metabolism in other, non-fructan, systems. However, much of the evidence to support the major hypotheses for its metabolism and regulation remains circumstantial, since purified proteins and clones for higher plant fructan genes have only recently become available.

There have been attempts to modify the reserve carbohydrate metabolism of other species by introducing both bacterial and higher plant genes associated with fructan metabolism and these experiments have generated plants with altered patterns of carbon partitioning, though more rigorous physiological examination will be required to substantiate some of the claims made on the basis of the available data. In terms of physiology, the current value of such transgenics is in terms of what they may tell us about the regulation of primary carbon metabolism in the recipient plants. Fructans are, in this case, only an alternative sink for carbon which may compete with existing metabolic pathways. In our view, transgenics currently provide little insight into the nature or control of endogenous fructan metabolism.

What is needed for the study of the endogenous metabolism is fructan-accumulating plants which have an altered capacity to make fructans. We propose that such plants would be of the highest scientific value if grass leaf metabolism was altered. The grass system has a number of advantages for such work: (a) the primary flux rates into sucrose and fructan are highest in these tissues, (b) their detailed physiology is extensively characterized, (c) bacterial levan is not hydrolyzed by grass FEH and would accumulate as a terminal sink, (d) the tissue has evolved to cope with fructan accumulation without deleterious effects, and (e) there is evidence from mutants and from inhibitor studies, that loss of endogenous fructan synthesis and the resultant accumulation of sucrose, are not immediately deleterious. Now that transformation of temperate Gramineae is routine, such an approach is feasible, as is the screening of species for mutations which alter fructan synthesis. The integration of current studies on enzymology and tissue compartmentation with the availability of such material will, we believe, provide the next major advance in the study of the regulation of fructan metabolism.
Acknowledgement

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Chapter 14

Acquisition and Diffusion of CO₂ in Higher Plant Leaves

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Summary

Acquisition of CO₂ by higher plants involves CO₂ diffusion from the air into leaves and, ultimately, into chloroplasts. There, fixation of CO₂ into organic compounds creates the concentration gradient which drives CO₂ diffusion. CO₂ encounters many obstructions along its diffusion path toward chloroplasts. The diffusion resistances attributable to boundary layer and stomata are shared with the opposing flux of water leaving the leaf. Once in the substomatal cavities, however, CO₂ faces additional resistances since it has to cross walls and membranes to reach the chloroplasts. We follow the CO₂ molecule from the air through the boundary layer, stomata and, finally, the mesophyll. After providing diverse anatomical examples at each level, we review the current understanding about the subtle balance which plants maintain between water loss and CO₂ acquisition.

Stomatal responses to many environmental variables are well known, despite our lack of understanding of the underlying mechanisms. Techniques are available that allow accurate measurements of conductances through the boundary layer and stomata. The estimation of conductance through the mesophyll, however, has not been feasible until the recent development of rapid measurements of isotopic discrimination and chlorophyll fluorescence. We discuss the principles which allow the estimation of internal conductance based on these techniques and the data available. Both stomatal conductance and internal conductance correlate strongly with photosynthetic capacity and are of similar magnitude. However, stomatal conductance can vary within minutes in response to changes in the environment. Internal conductance, on the other hand, seems to be stable over several days and depends on anatomical properties of the leaf. We close considering the special case of C₄ photosynthesis where spatial compartmentation and biochemical mechanisms of CO₂ concentration add complexity to the estimation of internal conductance.

I. Introduction

Photosynthesis requires light, CO₂ and water as substrates. Since water is usually a limited resource, plants control water losses by covering their aerial surfaces with impermeable structures which are perforated by stomata. Barriers to the diffusion of water also block the diffusion of CO₂. CO₂ and water share the same diffusion pathway from surrounding air to intercellular airspaces in the substomatal cavities. Consequently, any change to restrictions along the shared path will affect both CO₂ and water. Once inside the leaf, CO₂ faces additional diffusion barriers through intercellular airspaces, cell walls and cell matrix. In general, surrounding air is drier than air within leaves so that there is a net diffusion of water out of leaves. During photosynthesis, consumption of CO₂ inside mesophyll cells creates a partial pressure gradient that results in CO₂ diffusing into the plant. Thus the water and CO₂ fluxes are usually in opposition to one another.

Conventional gas exchange measures fluxes of water and CO₂ into and out of a leaf. The gradient in partial pressure of CO₂ from ambient air, p_{a}, to the substomatal cavities (usually referred to as the intercellular CO₂ partial pressure, p_i) is derived using Fick’s law of diffusion which states that the gradient in partial pressure is equal to the flux divided by the conductance p_{a} - p_{i} = A/g_s, where A is the rate of CO₂ assimilation and g_s is the stomatal conductance to CO₂. When considering fluxes, it is convenient to use conductances as these vary in proportion with the flux. However, for a pathway with a series of limitations, it is more convenient to use the reciprocal of conductance, resistance, as the total resistance for a pathway is simply the sum of the individual resistances (although for distributed sinks and mixed pathways, this is not strictly true (Parkhurst, 1994)).

This chapter explores the diffusion pathway from the air surrounding a leaf to the sites of CO₂ fixation within that leaf. Firstly, we consider the unstirred layer of air adjacent to the leaf surface which is...
called the boundary layer. This layer is influenced by
wind speed and such things as leaf size and shape as
well as smaller scale features such as grooves, crypts
and hairs. Secondly, we discuss stomata, the pores in
plant aerial surfaces that control resistance to diffusion
in response to many environmental signals. After
examining stomatal structure and distribution across
leaf surfaces of diverse plants, we discuss how they
respond to \( \text{CO}_2 \) leaf to air vapor pressure difference
and irradiance. The objective in this chapter is to
present the general features rather than provide a
comprehensive review of stomata which can be found
elsewhere (Farquhar and Sharkey, 1982; Zeiger et
al., 1987; Willmer and Fricker, 1996). There is a
striking correlation between stomatal conductance
and \( \text{CO}_2 \) assimilation rate that differs between \( \text{C}_3 \)
and \( \text{C}_4 \) plants. The mechanism behind these relationships
is not yet understood, but it results in an empirical
equation that can be used to predict stomatal
conductance. Gas exchange measurements usually
rely on an assumption that the leaf material enclosed
in the cuvette behaves uniformly. In some cases this
assumption has been shown to be false and we
discuss patchy or heterogeneous stomatal closure.

The third section considers internal leaf structure
where \( \text{CO}_2 \) encounters resistances not faced by water.
There are several methods available now to determine
internal resistance. Since these are relatively recent,
we present the methods and their strengths and
weaknesses in some detail. We then dissect the
problem of internal resistance into its airspace and
intracellular components. The fourth section
examines \( \text{C}_4 \) photosynthesis where anatomical and
biochemical specialization has enabled more efficient
\( \text{CO}_2 \) acquisition relative to water loss to be achieved.

II. Boundary Layer—\( \text{CO}_2 \) Diffusion to the
Leaf Surface

Adjacent to any surface is a layer of still air called the
boundary layer. When air moves past a leaf, air
nearest the leaf surface shears in parallel leading to
laminar flow near the leading edge. As one moves
away from the leading edge or away from the leaf
surface, air movement eventually becomes turbulent.
It turns out that the average thickness of this layer, \( l_b \),
can be approximated by \( l_b = 0.004 \sqrt{w / u} \) (m) where
\( w \) (m) is the width of the leaf in the direction of the air
movement and \( u \) (m s\(^{-1}\)) is the wind speed (Nobel,
1991). For example, the boundary layer thickness of
a tobacco leaf 150 mm wide in a 2 m s\(^{-1}\) wind would
be 1 mm whereas for a wheat leaf 20 mm wide it
would be only 0.4 mm thick. Boundary layer conductance,
\( g_b \), is then calculated as the ratio of the
diffusivity of the molecule in air and boundary layer
thickness. Boundary layer conductance is therefore a
function of wind speed which depends on leaf width
(Fig. 1). Narrow or dissected leaves have higher
boundary layer conductances than broad leaves.

Boundary layer conductance can also be influenced
by surface structures on the leaf and positioning of
stomata. It is common to find stomata in positions
away from the exposed leaf surface. They may be at
the base of grooves or pits, or the leaf could be rolled.
A dramatic illustration of this is seen with the desert
dwelling \( \text{C}_4 \) grass, spinifex (\( \text{Triodia irritans} \),
Fig. 2A,B). The leaf is rolled up so that it becomes
nearly circular in cross section. On both the exposed
and enclosed surfaces run parallel grooves about 100
\( \mu \text{m} \) deep which are lined with hairs. Stomata are only
found at the base of these grooves. The grooves are
aligned across the leaf, separated by a cluster of large
cells which by swelling or shrinking, can open and
close the gap in the grooves. If stomata in the grooves
around the outer surface were closed, \( \text{CO}_2 \) and water
molecules would be required to travel an additional
600 \( \mu \text{m} \) along the cleft.

Hairs are commonly found on leaf surfaces and
range considerably in density, length and shape. They play a clear role in increasing leaf reflectance during the summer in arid areas (Ehleringer and Björkman, 1978) or during leaf expansion (Ntefidou and Manetas, 1996). Hairs are also important for repelling water from the leaf surface during rain or dewfall (Brewer et al., 1991, Brewer and Smith, 1997). Since CO₂ diffusion through water is 10,000 times slower than through air, thin films of water on a leaf surface would effectively stop CO₂ assimilation. Hairs cause droplet formation on the leaf surface rather than a continuous film and can also raise the drops away from the leaf surface to further prevent occlusion of stomata. In dry conditions, the hairs trap air and increase the thickness of the boundary layer. Some hypostomatous leaves (those with stomata on only one surface) have a dense trichome layer above the surface with stomata and a smooth thick cuticle on the other surface which is generally the upper (adaxial) surface. This is illustrated by the leaf of the coastal New Zealand Christmas tree (Metrosideros thomasi, Fig. 2C,D). The lower (abaxial) surface is covered by a dense mat of curly hairs around 200 μm thick. At the base of these hairs protrude numerous stomata. The other surface has a thick cuticle and two layers of large cells beneath the

Fig. 2. Surface structures restricting CO₂ diffusion. The spiky leaf (Triodia irritans) (A, Craig and Goodchild, 1977; B, McWilliam and Mison, 1974) is rolled with hair-lined grooves that have stomata at their base. The large cells between pairs of grooves allow the groove aperture to widen or close with changes in turgor. The New Zealand Christmas tree (Metrosideros thomasi) (C,D, S Craig) has a dense layer of twisted hairs covering the lower (abaxial) surface which has stomata. The upper surface (panel D, leaf shown upside down) has no stomata, a thick cuticle and a double layer of cells beneath the epidermis that contains no chloroplasts.
epidermis devoid of chloroplasts.

The ratio of diffusivities of water and CO\textsubscript{2} are 1.37 in the boundary layer and 1.6 in stomata (von Caemmerer and Farquhar, 1981). Consequently, the ratio of conductances to CO\textsubscript{2} and water from surrounding air to sub-stomatal cavities depends to a small extent on the ratio of boundary layer to stomatal conductance. If boundary layer conductance is twice that of stomata, the ratio of conductance to CO\textsubscript{2} versus water is 0.66. This ratio increases to 0.71 if boundary layer conductance is only one quarter of stomatal conductance. That is, CO\textsubscript{2} uptake relative to water loss is favored when the boundary layer dominates the diffusion limitation, given equal leaf temperatures. However, since leaf temperature depends on boundary layer conductance and transpiration rate and transpiration rate depends on boundary and stomatal conductance, this is not a simple assumption.

When measuring gas exchange, leaf chambers are generally designed to have high windspeeds from either the flow rate or a fan so that boundary layer conductance is large relative to stomatal conductance.

### III. Stomata—CO\textsubscript{2} Diffusion into the Leaf

#### A. Leaf Surface Structure

Stomata vary in size, shape and density over the surface. Primitive leaves have stomata only on the lower surface (Mott et al., 1982) and a survey of British flora revealed that woody species also tend to be almost exclusively hypostomatus, with stomata only on the lower surface (Peat and Fitter, 1994). CO\textsubscript{2} entering the lower surface must diffuse across the bulk of these leaves because light is mainly absorbed at the upper surface. Generally, leaves with greater photosynthetic capacities, such as those that exist in sunny habitats, have additional stomata on their upper epidermis (amphistomatus), thus reducing the diffusion pathlength (Mott et al., 1982). Given the flexible and dynamic aperture of stomata, stomatal conductance is not well predicted by stomatal density (Jones, 1987).

Diverse examples of leaf surfaces are shown in Fig. 3. In panel A, a tobacco leaf is shown which is typical of many dicot herbaceous leaves. There is a high density of regularly spaced stomata with occasional protruding hairs. The surface of Kalanchoe (Fig. 3B), a CAM plant which has low stomatal conductance and nocturnal stomatal opening when water-stressed, clearly has a much lower stomatal density than tobacco. An apricot leaf is shown in Fig. 3C with a patch of stomata surrounded by veinlets. The veins have bundle sheath extensions reaching both epidermis and the epidermis above the veins is devoid of stomata. Nerium and Banksia leaves encrypt their stomata. Nerium leaves (Fig. 3D) have large pits scattered over the lower surface which are filled with hairs. Stomata are positioned near the base of the pits. Banksia (Fig. 3F, like Metrosideros Fig. 2C,D) has a dense array of hairs on the abaxial surface that obscure stomata from view. Wheat (Fig. 3E) is a typical monocot surface with stomata in linear files of cells, regularly spaced.

Another common feature is to place stomata at the base of an antechamber, such as in Pinus (Fig. 4). When viewed from the surface (Fig. 4C), the pores visible are not stomatal guard cells, but fixed lips of the antechamber. Inside the antechamber, wax structures place a further barrier to diffusion of water out and CO\textsubscript{2} in, before the stoma is reached (Fig. 4A). Beneath stomata are the sub-stomatal cavities (Fig. 4B) where CO\textsubscript{2} is free to diffuse away into the chlorenchyma through intercellular airspaces surrounding the highly lobed cells.

#### B. Stomatal Responses to CO\textsubscript{2}, VPD and Irradiance

Stomata begin to respond within seconds to changes in several environmental variables, reaching a new steady state after about 20 min (Kirschbaum et al., 1988). After prolonged darkness, initial stomatal opening may take longer than once the leaf is actively photosynthesizing. Their role is to enable CO\textsubscript{2} uptake while minimizing water loss (Cowan, 1977). Therefore, stomatal conductance tends to relate to CO\textsubscript{2} assimilation rate such that with increasing irradiance or decreasing CO\textsubscript{2}, stomatal conductance increases to maintain the supply of CO\textsubscript{2} needed for photosynthesis (Farquhar and Sharkey, 1982). On the other hand, stomatal conductance is also regulated in relation to leaf water status to control the rate of water loss. In the short-term, drier air surrounding the leaf which means a larger leaf to air vapor pressure difference, VPD, results in decreasing conductance. Stomatal responses of a given leaf are not fixed, but vary over the lifespan of the leaf (Constable and Rawson, 1980). If leaves have been water-stressed, stomatal sensitivity to CO\textsubscript{2} and VPD
increases (Raschke, 1987).

Steady-state responses to $\text{CO}_2$, VPD and irradiance are shown in Fig. 5. Because stomata respond to many signals, stomatal conductance is not uniquely related to any single variable. For example, when there is only a small leaf to air VPD, stomatal conductance is much greater at any given $\text{CO}_2$ concentration. The mechanism behind $\text{CO}_2$ respon-

Fig. 3. Leaf surfaces viewed by SEM, all at the same magnification. A, Tobacco (*Nicotiana tabacum*); B, *Kalanchoe diagremontiana* a CAM plant; C, Apricot (*Prunus persica*) – a heterobasic leaf; D, *Nerium oleander* – stomata are in the base of the hair-filled pits; E, Wheat (*Triticum aestivum*); F, *Banksia serrata* (Photos by D Bussis (A), K Maxwell (B), J Santrucek (C,D,F) and S Craig (E)).
siveness is as yet unknown. It appears that $\text{CO}_2$ is sensed via intercellular $\text{CO}_2$ rather than $\text{CO}_2$ in the stomatal pore or outside the leaf. Mott (1988) used amphistomatous leaves to examine the response of stomata on one surface where ambient and intercellular $\text{CO}_2$ could be independently varied. Stomatal conductance did not respond to changes in external $\text{CO}_2$ if intercellular $\text{CO}_2$ was kept constant, but did respond to changes in intercellular $\text{CO}_2$ when external $\text{CO}_2$ was kept constant.

Responses to VPD have been difficult to separate from those associated with evaporation rate and leaf temperature, since they all interact. However, in a clever experiment, Mott and Parkhurst (1991) substituted Helox (air in which $\text{N}_2$ is replaced by $\text{He}$) for air, which increases the rates of diffusion of water and $\text{CO}_2$ 2.3-fold over that in air. They followed stomatal aperture during step changes to Helox in which either evaporation rate or VPD was held constant. Stomatal aperture was unaltered by switching to Helox when evaporation rate was kept constant.}

**Fig. 4.** Pine needle. A, Stomatal complex fractured in cross section showing the guard cell pair with wax structures in the base of the antechamber; B, Sub-stomatal cavity directly below the stomata with two layers of chlorenchyma; C, Surface view of the needle showing the fixed pores of the antechambers. On the right hand side, the epidermis has been broken away revealing the mesophyll and regularly spaced substomal cavities. Multi-lobed cells in the mesophyll are lined with chloroplasts. (Photos by S Craig (A,B) and J Santrucce (C)).

**Fig. 5.** Stomatal conductance as functions of (A) ambient $\text{CO}_2$ concentration, (B) leaf to air vapor pressure difference (VPD) and (C) irradiance. Data from (A) Morison and Gifford (1983) for rice at different vpd; (B) Leuning (1995) *Eucalyptus grandis* at different temperatures; (C) Kirschbaum et al. (1988) *Alocasia macrorrhiza.*
constant by reducing VPD from 14.5 mmol mol$^{-1}$ in air to 6 mmol mol$^{-1}$ in Helox. By contrast, stomatal aperture was reduced following a switch to Helox when VPD was held constant. Initially, evaporation was much greater in Helox, but stomatal aperture declined until evaporation returned to the rate previously found in air. Mott and Parkhurst (1991) therefore concluded that stomatal responses to humidity or VPD are based on the rate of water loss from the leaf rather than sensing water vapor concentration at the leaf surface. Interestingly, gender specific responses to VPD have been observed for Box Elder (Acer negundo). Dawson and Ehleringer (1993) found that male trees were much more sensitive to VPD than female trees which resulted in stomatal closure and consequently lower intercellular CO$_2$ partial pressures in the males. This was true for sapling and mature trees when measured by gas exchange and was confirmed by carbon isotope composition.

Stomatal responses to irradiance consist of several sensing mechanisms, including a blue light response (Sharkey and Raschke, 1981) and another light response linked to photosynthesis. Over a wide range of irradiance, stomatal conductance increases proportionately with CO$_2$ assimilation rate (Wong et al., 1985b) such that intercellular CO$_2$ partial pressure remains constant. Investigating the mechanism for this again utilized amphistomatous leaves. By giving different combinations of irradiances to the two leaf surfaces, it was possible to uncouple conductance from irradiance for one surface. For example, the most extreme case was when light was only given to one surface. Stomatal conductance of the surface in the dark increased nearly three-fold when irradiance given to the other surface increased from 0.5 to 2 mmol quanta m$^{-2}$ s$^{-1}$ (Wong et al., 1985b). While $p_i$ for the abaxial surface increased slightly from 222 to 234 $\mu$bar, $p_i$ calculated for the whole leaf remained unchanged at 227 $\mu$bar. Thus, in this example overall conductance for the leaf was closely coupled to CO$_2$ assimilation rate and not the distribution of light between the two surfaces. This ruled out the possibility of stomatal conductance being controlled directly by irradiance under high irradiance conditions.

C. Relationship Between Stomatal Conductance and CO$_2$ Assimilation Rate

While stomatal conductance is responsive to several environmental variables, the parameter that correlates most closely with it is CO$_2$ assimilation rate. A close coupling has been found between stomatal conductance and rate of CO$_2$ assimilation regardless of whether CO$_2$ assimilation rate was varied by mineral nutrition, age or irradiance (Fig. 6, Wong et al., 1979, 1985a,b). Independent surveys have yielded striking correlations between $A$ and $g_s$ between species (Körner et al., 1979; Wong et al., 1979; Yoshie, 1986) with $C_3$ and $C_4$ species falling on two distinct lines.

The striking correlation between $A$ and $g_s$ (Fig. 6) for $C_3$ species implies a relatively constant ratio of intercellular to ambient CO$_2$ partial pressure, $p_i/p_a$. The ratio $p_i/p_a$ is negatively related to the ratio $A/g_s$, which defines the instantaneous water-use efficiency of photosynthesis (mol CO$_2$ fixed per mol water transpired, $A/E = (A/g_s)/(1.6VPD)$). Therefore, knowledge of $p_i/p_a$ has important ramifications in both agricultural and ecological work. Measurement of this ratio by conventional gas exchange techniques is labor intensive and it is difficult to resolve subtle differences between plants. However, with stable carbon isotope techniques (see Chapter 17, Brugnoli and Farquhar), it is now possible to screen plants using carbon isotopic composition, $\Delta$, as an integrated signal of $p_i/p_a$. For example, 351 $C_3$ Poa species were sampled in Africa and $^{613}$C ranged from –22 to –34‰ (Vogel, 1993) which is equivalent to a range in $p_i/p_a$ of nearly 0.5. More typically, $\Delta$ varies by around 2‰, equivalent to a range in $p_i/p_a$ of 0.1. Therefore, while $p_i/p_a$ is relatively conservative between $C_3$ species (0.7 ± 0.05), this implies greater variation in water-use efficiency as it is the same variation divided by 1 – $p_i/p_a$ (0.3 ± 0.05).

The mechanism behind the correlation between $A$ and $g_s$ is still not known, but in explaining their results, Wong et al. (1979) proposed that stomata must respond to a photosynthetic metabolite produced in the leaf mesophyll. However, Sharkey and Raschke (1981) used cyanazine or DCMU to inhibit photosynthesis and showed that stomatal conductance still responded to blue light. With the advent of transgenic plants, it has been possible to disrupt photosynthesis in leaves through the specific reduction of proteins by antisense gene expression. When CO$_2$ assimilation rate has been reduced via lowering the content of Rubisco (Hudson et al., 1992), GAPDH (Price et al., 1995) or cytochrome $b$/$f$ complex (Price et al., 1998), stomatal conductance was unaltered except in the most severely affected plants. Since it has been possible to break the correlation between $A$ and $g_s$, the role of a metabolite
Chapter 14 CO₂ in Plant Leaves

signal seems unlikely and the control mechanism remains a paradox. Despite our inability to unravel the mechanism, the strong correlation between \( A \) and \( g_s \) still provides us with the best predictor of stomatal conductance. Ball et al. (1987) empirically derived an equation that encapsulated stomatal responses to \( VPD \) and irradiance. It has subsequently been slightly refined (Leuning 1995):

\[
g_s = g_s^0 + a_v A / \left( (1 + D_v / D_o) \right)
\]

where \( g_s^0 \) is the conductance at zero irradiance, \( a_v \) and \( D_o \) are empirical coefficients, \( A \) is the rate of \( CO_2 \) assimilation, \( p_s \) is the partial pressure of \( CO_2 \) at the leaf surface, \( \Gamma \) is the \( CO_2 \) compensation point and \( D_v \) is the leaf to air \( VPD \). The problem with this relationship is that to find \( g_s^0 \), one first needs to know \( A \), which itself depends on \( g_s \) through its affect on intercellular \( CO_2 \). To obtain a solution therefore requires an iterative loop.

**D. Stomatal Patchiness**

Entire leaves or portions of a leaf are usually enclosed in cuvettes for gas exchange measurements. It is then assumed that the entire surface behaves uniformly when conditions are varied. Stomata may close in one section of a leaf and remain open in another, forming a patchy pattern. A comprehensive and erudite review was written by Terashima (1992) following his pioneering work in this field. Many leaves have densely packed sclerenchymatous cells that extend above and below vascular strands that effectively prevent lateral diffusion of gases. When these bundle sheath extensions are associated with reticulate venation, small patches of leaf are isolated from one another and the leaf is termed heterobaric. An example of a heterobaric leaf is apricot, shown in Fig. 3C. If lateral diffusion is unimpeded, the leaf is described as homobaric.

Much of the interest in patchy closure has been due to the consequences it has on interpretation of photosynthetic \( CO_2 \) response curves. If gas exchange averages across patches, it will underestimate \( CO_2 \) assimilation rate for a given intercellular \( CO_2 \). \( ABA \) has been associated with stomatal closure during water stress, but when applied to leaves, lowers both conductance and photosynthesis. It had been suggested that \( ABA \) directly inhibited photosynthesis, but this was shown to be an artifact of patchy stomatal closure (Terashima et al., 1988). Water stress has been found to cause patchy stomatal closure (Beyschlag et al., 1992; Eckstein et al., 1996, although see Gunasekera and Berkowitz, 1992) as well as exposure to large \( VPD \) (Loreto and Sharkey, 1990). While gas exchange revealed a steady conductance, fluorescence imaging showed patches of leaf oscillating widely, which when averaged out over a larger area, appeared stable (Cardon et al., 1994). Oscillations induced by a sudden drop in irradiance gradually dampen out, first adjacent to veins and gradually spreading out into the mesophyll (Siebke and Weis, 1995b).

There exist several methods for detecting the existence of patchy stomatal closure. Carbohydrate accumulation reflects the preceding photosynthesis which will be reduced if stomatal conductance was lower than in adjacent areas of a heterobaric leaf. Terashima et al. (1988) visualized this by subsequent iodine staining. They combined the visualization of photosynthate with direct observation of stomatal aperture on epidermal replicas of the same leaf pieces. Areas with little starch staining corresponded to areas with closed stomata. Downton et al. (1988) used \( ^{14}CO_2 \) labeling and autoradiography to visualize the spatial pattern of photosynthesis. More recently, chlorophyll fluorescence has been used to resolve the spatial and temporal patterns of photosynthesis.
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(Daley et al., 1989) and offers superb resolution. An alternative approach has been to assess stomatal conductance by measuring the infiltration of leaves by liquids of different viscosity (Beyschlag and Pfanz, 1990). Comparisons between conductance measured by gas exchange and quantity of water infiltrated into the same leaf yielded proportional linear relationships (Beyschlag and Pfanz, 1990; Beyschlag et al., 1992).

Clearly, heterogeneous stomatal closure is a widespread phenomenon that occurs for multiple reasons. However, this does not mean it is a common occurrence that always plagues gas exchange analysis. With unstressed plants not subject to abrupt changes in light or VPD, or for homobaric leaves, patchy stomatal closure is unlikely to be encountered.

IV. Internal—\(\text{CO}_2\) Diffusion Within the Leaf

A. Leaf Mesophyll Structure

Just as there is great diversity in the surface of leaves in terms of hairs and patterns of stomata, so there is with mesophyll structure (Fig. 7, see also Bolhar-Nordenkampf and Draxler, 1993). The examples presented in Fig. 7 are all shown at the same magnification. Perhaps the most common feature is palisade tissue near the leaf surface. These cylindrical cells are closely packed in parallel to one another directly beneath the leaf surface. For bifacial leaves, palisade cells are usually beneath the adaxial surface, with spongy mesophyll near the abaxial surface (Fig. 7 A, B, C). Huge, densely packed cells fill the thick succulent tissue of the CAM plant \textit{Kalanchoe} of which only a small fraction is shown in Fig. 7D. Monocots have cell shapes that depend on their position relative to vascular strands (Fig. 7E). For leaves that hang or grow vertically, isolateral anatomy is often found where palisade tissue is present on both surfaces with spongy tissue in the central part of the leaf (Fig. 7F). With the thick horizontally displayed leaf of \textit{Metrosideros} (Fig. 7G), densely packed cells are present throughout the mesophyll.

It is difficult to fully appreciate intercellular airspaces from transverse fractures or sections, but clearly the airspaces of spongy mesophyll offer little resistance to diffusion. In palisade tissue, narrow pores extend up between adjacent cells and a cavernous gap can be seen near the top of Fig. 7D in the densely packed succulent tissue of the CAM leaf. When the scanning electron microscopy was done with ‘nude’ (i.e. uncoated) samples, it is possible to see chloroplasts through the cell walls (Fig. 7 A, C, D). Chloroplasts cover the cell surface in tobacco and cotton (Fig. 7A, C) but are scattered diffusely over the surface of the large balloon-like cells of the CAM plant \textit{Kalanchoe} (Fig. 7D, Maxwell et al., 1997). For coated samples, the chloroplasts are only evident when a cell is fractured open. Chloroplasts are then seen adjacent to the cell walls (Fig. 7E).

B. Methods for Determining Internal Conductance

Diffusion of \(\text{CO}_2\) from substomatal cavities, \(p_i\), to sites of carboxylation, \(p_o\), must occur down a partial pressure gradient. Opinion as to the magnitude of this gradient has swung from one extreme (e.g. Gaasstra, 1959) that \(p_o = 0\) to the other (e.g. Farquhar and von Caemmerer, 1982) that \(p_o\) is approximately equal to \(p_i\). The ease with which \(\text{CO}_2\) can diffuse through the leaf to the sites of carboxylation can be defined by an internal conductance, \(g_w\), using Fick’s law: \(g_w = A / (p_o - p_i)\). The subscript \(w\) was introduced by Evans (1983b) to emphasize the wall and liquid phase components of internal conductance. There exist several methods for determining \(g_w\) which are described below.

1. Stable Isotope Discrimination Combined with Gas Exchange

There are two naturally occurring stable isotopes of carbon, \(^{13}\text{C}\) and \(^{12}\text{C}\). About 1.1% of atmospheric \(\text{CO}_2\) contains the heavier isotope \(^{13}\text{C}\), \(^{13}\text{CO}_2\) diffuses more slowly than \(^{12}\text{CO}_2\) and has slightly different chemical properties. Consequently, the carbon isotopic composition of plant material is depleted of \(^{13}\text{C}\) relative to atmospheric \(\text{CO}_2\). Not only does \(^{13}\text{CO}_2\) have a lower diffusivity in air than \(^{12}\text{CO}_2\), but because Rubisco discriminates against \(^{13}\text{CO}_2\), there is also a smaller difference in partial pressure. The preferential fixation of \(^{12}\text{CO}_2\) during photosynthesis results in an enrichment of \(^{13}\text{CO}_2\) in air surrounding the leaf.

Theory has been developed (Farquhar et al., 1982; Farquhar and Richards, 1984) that predicts net discrimination, \(\Delta\), by \(C_4\) plants:
Fig. 7. Leaf mesophyll structure compared at the same magnification. A, Tobacco (*Nicotiana tabacum*); B, Banksia marginata; C, Cotton (*Gossypium hirsutum*); D, Kalanchoe diuricriontiana – only a small portion as the leaf thickness was 1.2 mm; E, Wheat (*Triticum aestivum*); F, Eucalyptus pauciflora; G, Metrosideros thomasi. Photos by S Craig (B,E,G), J Santrucek (C) and K Maxwell (D).
where \( \alpha_i \) and \( a \) are the fractionations during diffusion through air in the boundary layer and stomata, respectively (2.9‰, 4.4‰), \( a_i \) is the combined fractionation due to dissolution and diffusion of \( \text{CO}_2 \) in water (1.8‰), \( b \) is the fractionation during carboxylation that occurs during photosynthesis, \( e \) and \( f \) represent fractionations associated with nonphotorespiratory \( \text{CO}_2 \) evolution, \( R \), and photorespiration, respectively, \( k \) is the carboxylation efficiency of Rubisco and \( \Gamma \), is the \( \text{CO}_2 \) compensation point in the absence of dark respiration (Farquhar et al., 1982) and \( p_{\text{a}}, p_{\text{s}}, p_{\text{c}}, \text{and } p_{\text{e}} \) are the partial pressures of \( \text{CO}_2 \) in the surrounding air, leaf surface, intercellular airspace (strictly speaking, the substomatal cavity) and sites of carboxylation, respectively. The value of \( b \) has been determined in vitro (30 - 31.3‰, Roeske and O’Leary, 1984, Guy et al., 1993), but in the leaf a small proportion of \( \text{CO}_2 \) is fixed via phosphoenol pyruvate carboxylase which reduces the effective value of \( b \) in vivo (Farquhar and Richards, 1984). From concurrent measurements of gas exchange and discrimination in tobacco leaves that have an antisense RNA gene for Rubisco small subunit an estimate of 29‰ was obtained (Evans et al., 1994). Attempts to measure fractionation during respiration have suggested that \( e \) is close to zero (von Caemmerer and Evans, 1991). There appears to be significant fractionation during photorespiration \( (f \approx 8\%), \text{ Rooney, 1988; } 3 - 12\%, \text{ Gillon and Griffiths, 1997).} \)

It is usual to make some simplifications to Eq. (1) and present the relationship as

\[
\Delta_i = \frac{a}{p_a} \left( \frac{p_a - p_s}{p_a} \right) + \frac{a}{p_a} \left( \frac{p_s - p_i}{p_a} \right) + \frac{a_i}{p_a} \left( \frac{p_i - p_c}{p_a} \right) + b \frac{p_e}{p_a} \left( \frac{eR}{k + f\Gamma} + \frac{i}{\gamma^*} \right) \tag{1}
\]

This form of the equation distinguishes between fractionation during diffusion through air (\( a \)) and during carboxylation (\( b \)) with discrimination being linearly dependent on \( p / p_a \). It is based on several assumptions. 1. The drawdown across the boundary layer and stomata are combined as this introduces little error and usually boundary layer conductance is not routinely measured. 2. The drawdown from substomatal cavities to sites of carboxylation is negligible or constant. 3. Fractionations associated with dark respiration and photorespiration are negligible. One can rearrange Eq. (2) to highlight that \( \Delta \) is simply related to the balance between \( \text{CO}_2 \) assimilation rate, \( A \), and leaf conductance, \( g^* \):

\[
\Delta_i = b - (b - a)A/(g^* p_a). \tag{3}
\]

During photosynthesis, \( \text{CO}_2 \) passing over the leaf changes its isotopic composition. By cryogenically trapping \( \text{CO}_2 \) from the source air and \( \text{CO}_2 \) from air that exits a gas exchange chamber (‘on-line’) and subsequently measuring their isotopic composition, it is possible to derive the net discrimination that occurred during that photosynthetic state (Evans et al., 1986, von Caemmerer and Evans, 1991). Multiple measurements can then be made on a leaf under a variety of conditions and subsequently be related to the photosynthetic measurements. This has enabled examination of the drawdown from substomatal cavity to the sites of carboxylation. By subtracting Eq. (1) from Eq. (2), one obtains:

\[
\Delta_i - \Delta = (b - a_i) \frac{(p_i - p_c)}{p_a} + \frac{eR}{k + f\Gamma} + \frac{i}{\gamma^*} \tag{4}
\]

Equation 4 reveals that the difference between the discrimination predicted on the basis that \( p_i = p_e, \Delta_i \), and the measured \( \Delta \) should be linearly related to \( A/p_a \) with the slope proportional to the reciprocal of the internal conductance, \( g^* \). The second term containing the respiration and photorespiration terms should appear as a non-zero intercept.

An example of this technique is shown in Fig. 8. A radish leaf was measured at a range of irradiances beginning at 1.35 mmol quanta m\(^{-2}\) s\(^{-1}\), decreasing to 0.11 mmol quanta m\(^{-2}\) s\(^{-1}\) and returning back to the starting irradiance. The insert shows the measured \( \Delta \) as a function of \( p / p_a \), with \( p / p_a \) increasing slightly at the lowest irradiances. The solid line represents \( \Delta_i \) (Eq. 2) and the difference between \( \Delta \) and \( \Delta_i \) is replotted as a function of \( \text{CO}_2 \) assimilation rate in the main panel. The strong linear increase in difference between \( \Delta \) and \( \Delta_i \) gives clear support to the notion that there is a significant difference between \( p_i \) and \( p_e \) which...
depends on the rate of CO$_2$ assimilation. The data are consistent with a resistance that is fixed over the measurement time period for a given leaf. Repeated measurements on a tobacco leaf over the course of a week have shown that the internal conductance did not change (Evans and von Caemmerer, unpublished). The non-zero intercept reveals that the fractionations associated with respiration and photorespiration are occurring, but it also contains experimental error. Consequently, an estimate of drawdown to the sites of carboxylation made from a single measurement is considerably less certain than that obtained by determining the slope as in Fig. 8 which is quite robust.

While this technique has been used to study internal conductance, it requires access to a ratio mass spectrometer with measurement precision an order of magnitude better than can be tolerated for plant dry matter samples. Since these machines are rare, the technique is not readily available for many people. The alternative is to determine $\Delta$ on soluble sugars extracted from a leaf that has been held under constant conditions during photosynthesis and for which gas exchange parameters are known. One can then apply Eq. (4). The $\Delta$ of soluble sugars is known to closely reflect $p_i/p_o$ (Brugnoli et al., 1988) and has been used to calculate $g_w$ in rice (Sasaki et al., 1996) and sunflower, cotton and chestnut (Brugnoli et al., 1998). While the method is far less precise than on-line measurements, because it depends on the value assumed for $b$ and assumes that the second term in Eq. (4) is zero, it should prove useful when a relative value is being sought. If access to ratio mass spectrometry is not possible, an alternative method is to combine gas exchange with chlorophyll fluorescence measurements.

2. Chlorophyll Fluorescence Combined with Gas Exchange

Development of the modulated chlorophyll fluorometer has enabled fluorescence to be measured on leaves during photosynthesis. All that is required are two measurements, namely steady state fluorescence, $F$, followed by fluorescence during a pulse of light (generally several times full sunlight) that closes all Photosystem II reaction centers, $F_m$. Photochemical efficiency of Photosystem II, that is, the proportion of quanta absorbed by Photosystem II that result in electron transport, is given by (Genty et al., 1989),

$$\phi_{PSII} = 1 - F/F_m.$$

(5)

Although this measurement appears simple, to use it to calculate the rate of electron transport requires important assumptions or calibration for a particular leaf. The rate of electron transport calculated from fluorescence, $J_F$, is given by,

$$J_F = \phi_{PSII} \cdot I \cdot a \cdot \beta,$$

(6)

where $I$ is the irradiance, $a$ is the leaf absorptance (generally around 0.8–0.85) and $\beta$ is the fraction of absorbed quanta that reach PS II (generally 0.42–0.5). Equation (6) can be obtained by calibration. Fluorescence and gas exchange measurements are made at very low oxygen partial pressures that prevent photorespiration. The rate of electron transport, $J$, that is required to support a given rate of CO$_2$ assimilation, $A$, is given by (Farquhar and von Caemmerer, 1982),
\[ J = (A + R)(4p_c + 8\Gamma_*) / (p_c - \Gamma_*), \]

where \( \Gamma_* \) is the CO₂ photocompensation point in the absence of nonphotorespiratory CO₂ evolution, \( R \) is linearly related to oxygen partial pressure, so in the limit when there is no oxygen present, Eq. (7) simplifies to \( J = 4(A + R) \). Some uncertainty remains because \( R \) is partially suppressed in the light, although it is relatively constant over a broad range of irradiances above 0.2 mmol quanta m\(^{-2}\)s\(^{-1}\) (Brooks and Farquhar, 1985). For a given leaf, \( J_c \) is measured over a range of irradiances and or CO₂ partial pressures to establish the relationship between \( J/I \) and \( \phi \text{PSII} \). A linear relationship should be observed with the slope equivalent to \( a \beta \) (Siebke and Weis, 1995a; Genty and Meyer, 1995). In some instances, a non-zero intercept is observed suggesting that not all electron transport is associated with photosynthesis (Laisk and Loreto, 1996), but this problem and the uncertainty in \( R \) become less important at higher irradiances.

Having established the relationship between \( J \) and \( \phi \text{PSII} \) in Eq. (6), the combined measurement of fluorescence and gas exchange measurements under 21% oxygen over a range of CO₂ partial pressures or irradiances is used to calculate \( p_c \) which is given by rearranging Eq. (7),

\[ p_c = \Gamma_* (J_c + 8(A + R)) / (J_c - 4(A + R)). \]

Internal conductance can then be calculated for each point from the definition, \( g_m = A/(p_c - p_x) \).

An example of the method is given for control and water-stressed spinach leaves (Fig. 9). Electron transport rate derived from fluorescence is shown as functions of irradiance (Fig. 9A squares). At higher irradiance, electron transport rate was calculated from gas exchange using Eq. (7) assuming \( p_i = p_c \) (triangles) or calculating \( p_i \) as \( p_i = A/g_m \) and assuming \( g_m = 0.63 \) or 0.18 mol m\(^{-2}\)s\(^{-1}\)bar\(^{-1}\) for control and water-stressed leaves, respectively, (circles). In Fig. 9B the rate of CO₂ assimilation is plotted as a function of the drawdown \( p_i - p_c \), where \( p_i \) is obtained from gas exchange and \( p_c \) is calculated from chlorophyll fluorescence using Eq. (8). The data conform to the expectation that drawdown is proportional to the rate of CO₂ assimilation and the slope yields the estimate of internal conductance. Water stress clearly reduced internal conductance, as has been observed in other studies (Renou et al., 1990; Roupsard et al., 1996; Ridolfi and Dreyer, 1997; Brugnoli et al., 1998). Salt stress has also been shown to reduce internal conductance in olive (Bongi and Loreto, 1989) and spinach leaves (Delfine et al., 1998).

The first to combine fluorescence with gas exchange to determine \( p_c \) was Di Marco et al. (1990). The method was subsequently refined by Harley et al. (1992) who proposed two modes of calculating internal conductance. The first, coined the `variable J’ method (Shown in Fig. 9), calculates \( p_c \) from Eq. (8) for a series of measurements where variation in either CO₂ or irradiance results in changes to the rate
of electron transport calculated from chlorophyll fluorescence. The individual estimates are then averaged. In the second, coined the ‘constant J’ method, a series of measurements are made at an intermediate irradiance over a range of CO$_2$ partial pressures which do not alter the rate of electron transport calculated from chlorophyll fluorescence. A series of calculations are then made using Eq. (7) where different values for the internal conductance are assumed in order to calculate $p_c$. The minimum variance in the calculated J for the set of measurements is then sought and taken as the best estimate of internal conductance.

Harley et al. (1992) examined the limitations and potential errors associated with uncertainties in the various parameters. Perhaps the most crucial is $\Gamma_i$. $\Gamma_i$ can either be measured by gas exchange techniques (Laisk, 1977, Brooks and Farquhar, 1985) or calculated from the specificity factor of Rubisco determined in vitro. There are now a considerable number of values reported for $\Gamma_i$ in a range of species (Table 1). There is no consistent difference between species, given the scatter for each. In order to illustrate the significance of uncertainty in calculating $g_w$, the data presented in Fig. 9 has been analyzed using different values of $\Gamma_i$ and $R$ (Table 2). The two values chosen for $\Gamma_i$ were 42.7 μbar (Brooks and Farquhar, 1985) and 38.6 μbar (von Caemmerer et al., 1994). For the water-stressed leaf, variation in $R$ and $\Gamma_i$ did not greatly affect the estimate of $g_w$. However, the estimate for the control leaf varied a great deal. This illustrates that this method becomes increasingly uncertain as $g_w$ increases as was pointed out by Harley et al. (1992). When $g_w$ is greater than 0.3 mol m$^{-2}$s$^{-1}$ bar$^{-1}$, it would be advisable to measure $\Gamma_i$ and $R$ for the leaf. The normal protocol for obtaining the photocompensation point is the point of intersection of CO$_2$ response curves measured at different irradiances. The CO$_2$ partial pressure at this point, $p_i^*$ and the rate of CO$_2$ evolution, $R$, are then used to calculate $\Gamma_i$, as follows (von Caemmerer et al., 1994):

$$\Gamma_i = p_i^* + R/g_w$$

(9)

$\Gamma_i$ is greater than $p_i^*$ because mitochondrial

Table 1. $\Gamma_i$ values in the literature converted to 25 °C
(* values were measured in vitro, all the others were measured in the leaf by gas exchange and in some cases with fluorescence as well)

<table>
<thead>
<tr>
<th>Species</th>
<th>$\Gamma_i$ (μbar)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinacia</td>
<td>47.7$^A$</td>
<td>Jordan and Ogren (1984)</td>
</tr>
<tr>
<td></td>
<td>42.1</td>
<td>Brooks and Farquhar (1985)</td>
</tr>
<tr>
<td></td>
<td>33.6</td>
<td>von Caemmerer et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>48.2–55.2$^A$</td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td>Nicotiana</td>
<td>38.1$^A$</td>
<td>Parry et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>46.6</td>
<td>Peterson (1990)</td>
</tr>
<tr>
<td></td>
<td>44.2$^A$</td>
<td>Delgado et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>38.6</td>
<td>von Caemmerer et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>48.4$^A$</td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td>Triticum</td>
<td>39</td>
<td>Brooks and Farquhar (1985)</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>Watanabe et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>44.1$^A$</td>
<td>Kane et al. (1994)</td>
</tr>
<tr>
<td>Helianthus</td>
<td>43.1–46.1</td>
<td>Laisk and Sunberg (1994)</td>
</tr>
<tr>
<td></td>
<td>35.8</td>
<td>Epron et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>44.6</td>
<td>Laisk and Loreto (1996)</td>
</tr>
<tr>
<td>Phaseolus, Populus, Viburnum various</td>
<td>43.8</td>
<td>Laisk (1977)</td>
</tr>
<tr>
<td>Heteromeles, Lepechinta</td>
<td>36.9–43.2</td>
<td>Berry et al. (1994)</td>
</tr>
<tr>
<td>Phaseolus</td>
<td>43.9, 43.4</td>
<td>Villar et al. (1994)</td>
</tr>
<tr>
<td>Hordeum</td>
<td>37.4</td>
<td>Epron et al. (1995)</td>
</tr>
<tr>
<td>Poa (6 species)</td>
<td>34.1</td>
<td>Hauser et al. (1996)</td>
</tr>
<tr>
<td>Quercus</td>
<td>33.1–42.6</td>
<td>Atkin (pers. com.)</td>
</tr>
<tr>
<td></td>
<td>38.8, 42.5$^A$</td>
<td>Balaguer et al. (1996)</td>
</tr>
</tbody>
</table>

Table 2. The effect of varying $\Gamma_i$ and $R$ on the estimate of $g_w$ in Fig. 9

<table>
<thead>
<tr>
<th>$\Gamma_i$ (μbar)</th>
<th>42.7</th>
<th>42.7</th>
<th>38.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$ (μmol m$^{-2}$s$^{-1}$)</td>
<td>1</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>$g_w$ (mol m$^{-2}$s$^{-1}$ bar$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.88</td>
<td>0.63</td>
<td>0.36</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>0.19</td>
<td>0.18</td>
<td>0.14</td>
</tr>
</tbody>
</table>

(1985) and 38.6 μbar (von Caemmerer et al., 1994).
respiratory efflux increases CO₂ within the chloroplast above that in the substomatal cavities. Another uncertainty with the variable J method is the fraction of electrons that flow to sinks other than photosynthesis (Laisk and Loreto, 1996). If there are other sinks, fluorescence tends to overestimate the electron transport rate and consequently underestimates \( g_w \).

### 3. \(^{18}\text{O}\) Uptake in Combination with Gas Exchange

The uptake of O₂ by a leaf includes three processes: photorespiration, O₂ photoreduction (Mehler reaction) and dark respiration. The total O₂ uptake can be measured mass spectrometrically by using \(^{18}\text{O}\) (Renou et al., 1990). Net CO₂ uptake, on the other hand, is gross photosynthesis less carbon losses in photorespiration and dark respiration and can be measured by CO₂ gas exchange.

This method for calculating \( g_w \) is based on the comparison of CO₂ and O₂ uptake and assumes that: 1. O₂ photoreduction is negligible and 2. nonphotorespiratory CO₂ evolution, \( R \), is known. Oxygen uptake \( (U_o) \) is given by the sum of velocity of oxygenation \( (v_o) \), oxygen consumed in the oxidation of glycolate \( (0.5v_o) \) and nonphotorespiratory CO₂ evolution, \( R \):

\[
U_o = v_o + 0.5v_o + R
\]

\[
v_o = 2/3(U_o - R).
\]

CO₂ assimilation rate, \( A \), equals rate of carboxylation \( (v_c) \) minus half the rate of oxygenation \( (0.5v_o) \) and \( R \):

\[
A = v_c - 0.5v_o - R
\]

\[
v_c = A + U_o/3 + 2R/3.
\]

Just as in the fluorescence method, it is necessary to use \( \Gamma_x \). By definition (Farquhar and von Caemmerer, 1982),

\[
p_c = 2 \times \Gamma_x / (v_o/v_c)
\]

\[
= \Gamma_x \times (3A + U_o + 2R) / (U_o - R).
\]

From the water and CO₂ exchange measurements, \( p_i \) is known, which enables \( g_w \) to be calculated from the definition, \( g_w = A / (p_c - p_i) \).

This method has been successfully used to investigate the drawdown between \( p_i \) and \( p_c \) following water stress in wheat (Renou et al., 1990) and to examine the role of carbonic anhydrase in Commelina (Peltier et al., 1995). A drawback of the method was that whole shoots were enclosed in the chamber leading to uncertainties in both leaf temperature (necessary for calculating \( p_i \) and \( R \)). The use of \(^{18}\text{O}\) and the need for a mass spectrometric system limits the availability of this method.

### 4. Comparison of the Methods for Measuring Internal Conductance

Three different techniques for measuring \( g_w \) have been described. Each requires a parameter to be measured in addition to conventional gas exchange in order to assess the restriction to CO₂ diffusion within the leaf. Loreto et al. (1992) have carried out what appears to be the only comparison between methods so far. They compared four leaves of both Quercus rubra and Xanthium strumarium using carbon isotope discrimination and chlorophyll fluorescence methods. For Quercus, which had a lower photosynthetic capacity than Xanthium, the different methods all yielded values of \( g_w \) around 0.15 mol m⁻² s⁻¹ bar⁻¹. For Xanthium, isotope discrimination yielded a value of 0.49 mol m⁻² s⁻¹ bar⁻¹ which was 20% less than the variable J method, while the constant J method failed to yield a minimum variance. To incorporate other leaves where just one method had been used, Loreto et al. (1992) also examined the relationship between \( g_w \) and photosynthetic rate at ambient CO₂ and high irradiance (see also below in section C). Again, there was close agreement between the methods for leaves with values of \( g_w \) below 0.3 mol m⁻² s⁻¹ bar⁻¹ and the fluorescence methods yielded higher values than carbon isotope discrimination for leaves with \( g_w \) above 0.3 mol m⁻² s⁻¹ bar⁻¹. The fact that independent methods yield similar values for \( g_w \) is strong evidence that the magnitude of the diffusion gradient within the leaf is accurate.

One can also compare the methods on the basis of their reliability and ease of use. If an absolute value is required, the carbon isotope discrimination method with measurements made over a range of irradiances is undoubtedly the most robust. Single measurements are to be avoided as they introduce uncertainty due to the discrimination associated with photorespiration and other experimental errors. Unfortunately, this method is not readily available as it requires access to a high precision mass spectrometer. Therefore, it is...
more likely that one of the chlorophyll fluorescence methods would be chosen. These methods work best for leaves with values of \( \gamma \) below 0.3 mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\). The constant J method only uses chlorophyll fluorescence to indicate the range of CO\(_2\) over which electron transport is constant. It is therefore more robust than the variable J method which requires that the chlorophyll fluorescence signal is calibrated and assumptions about alternative electron acceptors.

If one is only interested in comparison between cultivars or species, then measurement of the carbon isotopic composition of source air and of extracted sugars or dry matter in combination with gas exchange measurements to determine \( p_\text{e} \) may be acceptable. It is important to sample source air when plant material is grown indoors, in glasshouses or with CO\(_2\) supplementation. A simple method is to grow C\(_4\) plants alongside the material of interest. The isotopic signal in extracted sugars is more likely to reflect current photosynthesis and the estimate of \( p_\text{i} \) than that in dry matter. While this technique is less accurate, it is more accessible because the carbon isotopic composition can be readily made in many laboratories.

C. Relationship Between Internal Conductance and CO\(_2\) Assimilation Rate

There have now been a range of species where \( g_\text{w} \) has been measured either by \( A \) or fluorescence methods. What has emerged is a striking correlation between the photosynthetic capacity and internal conductance of a leaf (Fig. 10A). A line across the diagonal yields an average drawdown from \( p_\text{i} \) to \( p_\text{e} \) of 78 \( \mu \)bar, only slightly less than the drawdown from \( p_\text{a} \) to \( p_\text{e} \) (Fig. 6). Mesophytic and sclerophyllous leaves are distinguished by open and solid symbols, respectively, to emphasize that they share a common relationship. In general, sclerophyllous leaves have lower photosynthetic capacities as well as lower internal conductances. Since the ratio \( A/g_\text{w} \) is similar for both sclerophyllous and mesophytic leaves, the drawdown from substomatal cavities to sites of carboxylation is similar in both leaf types (Fig. 10B). That is, low internal conductance does not mean that \( p_\text{e} \) will be low. If \( p_\text{i} \) is low, this may well be due to low stomatal conductance. It is time to correct the mistaken impression that has emerged in the literature that woody species are more disadvantaged by low internal conductances. This has been suggested by both Lloyd et al. (1992) and Epron et al. (1995), despite the fact that their data shows that the ratio \( A/g_\text{w} \) for woody species is similar to that for herbaceous leaves. While it is true that there was a trend for \( p_\text{e} \) to be lower for the leaves with lower photosynthetic rates (Lloyd et al., 1992), this was due to lower \( p_\text{i} \) because the difference between \( p_\text{i} \) and \( p_\text{e} \) was independent of photosynthetic capacity. Epron et al. (1995) stated
that ‘high internal resistances to CO₂ transfer may account for the low net CO₂ assimilation rates that often characterize tree leaves’. However, low assimilation rate reflects the low biochemical capacity, not a high internal resistance. If high internal resistance was the cause of low CO₂ assimilation rates, pᵣ – pₑ would be much greater for sclerophytic leaves and this is not observed (Fig. 10B).

Over the lifespan of the leaf, both photosynthetic capacity and gₛ change. Studies with wheat have shown that as leaves age, both photosynthetic capacity and gₛ decline in parallel (Loreto et al., 1994; Evans and Vellen, 1996; Fig. 10). The changes are slow, occurring over periods of days to weeks, reflecting the remobilization of photosynthetic proteins such as Rubisco. The factors that may determine gₛ are discussed below in section D, but it is not yet clear what factors are responsible for the decline in gₛ as leaves age. The relative stability of gₛ contrasts with that of stomatal conductance which can respond in seconds to minutes. Another intriguing observation is the suggestion that gₛ might be sensitive to the growth temperature in rice (Makino et al., 1994), something which has yet to be followed up.

D. Determinants of Internal Conductance

CO₂ assimilation is distributed between chloroplasts and cells within the leaf. This means that the use of the Ohm’s law analogy for resistances in series is prone to error. Parkhurst (1994) has examined in great detail how this analogy does not provide an adequate description of intercellular CO₂ partial pressure. The pattern of CO₂ uptake and distribution of photosynthetic capacity through the leaf can also bias the calculation of internal conductance (Lloyd et al., 1992). Bearing these problems in mind, it is still useful to consider the internal resistance as the sum of the intercellular airspace resistance, rᵢₘ, and the liquid phase resistance, rᵢₗₐₗ, such that

\[ r_w = rᵢₘ + rᵢₗₐₗ \text{ or } g_w = \frac{gᵢₗₐₗ}{1 + \frac{gᵢₗₐₗ}{gᵢₘ}} \]  \hspace{1cm} (13)

Chloroplasts cover the majority of the exposed cell surface area in tobacco and because diffusion through liquid in the absence of carbonic anhydrase is slow, CO₂ is unlikely to enter the chloroplast from the side unless it is derived from respiration and photorespiration. Therefore, conductance across the cell wall and chloroplast can be scaled up to a projected leaf area basis by multiplying by the exposed chloroplast surface area per unit leaf area, Sₑ. That is,

\[ gᵢₗₐₗ = gᵢₗₐₗₑ Sₑ \]  \hspace{1cm} (14)

where gᵢₗₐₗₑ is the conductance across the cell wall and chloroplast per unit exposed chloroplast surface area. Substituting Eq. (14) into Eq. (13) we obtain

\[ g_w = \frac{gᵢₗₐₗₑ · Sₑ}{1 + \frac{gᵢₗₐₗₑ}{gᵢₘ}} \]  \hspace{1cm} (15)

If gᵢₘ ≫ gᵢₗₐₗₑ we can approximate Eq. (15) by,

\[ g_w = gᵢₗₐₗₑ · Sₑ \]  \hspace{1cm} (16)

Thus it can be seen that gₛ is proportional to Sₑ if the airspace conductance is large relative to gᵢₗₐₗₑ or proportional to gᵢₗₐₗₑ · Sₑ.

1. Intercellular Airspace Resistance

Having reached the sub-stomatal cavity of a leaf, CO₂ needs to diffuse throughout the intercellular airspaces to reach cell wall surfaces adjacent to chloroplasts. In bifacial leaves with spongymosphyll tissue on the abaxial (lower) surface, intercellular airspaces are large (Fig. 7). This should offer little resistance to CO₂ movement apart from the distance traveled. Palisade tissue on the adaxial (upper) surface is much more ordered and can be tightly packed. Here, CO₂ diffusion must occur through narrow gaps alongside adjacent cells. Most CO₂ needs to reach this tissue as it is this part of the leaf that dominates CO₂ assimilation (Nishio et al., 1993).

Amphistomatous leaves which have stomata on both the upper and lower surfaces make it possible to independently measure the substomatal CO₂ partial pressure of each surface. Sharkey et al. (1982) measured leaves of Xanthium and Gossypium in order to validate the technique used to calculate CO₂ assimilation (Nishio et al., 1993). The difference between calculated CO₂ assimilation and measured CO₂ assimilation ranged between 0 and 9 μmol CO₂ cm⁻² s⁻¹. Sharkey et al. (1982) measured leaves of Xanthium and Gossypium in order to validate the technique used to calculate CO₂ assimilation (Nishio et al., 1993). The difference between calculated CO₂ assimilation and measured CO₂ assimilation ranged between 0 and 9 μmol CO₂ cm⁻² s⁻¹. Sharkey et al. (1982) measured leaves of Xanthium and Gossypium in order to validate the technique used to calculate CO₂ assimilation (Nishio et al., 1993). The difference between calculated CO₂ assimilation and measured CO₂ assimilation ranged between 0 and 9 μmol CO₂ cm⁻² s⁻¹. Sharkey et al. (1982) measured leaves of Xanthium and Gossypium in order to validate the technique used to calculate CO₂ assimilation (Nishio et al., 1993). The difference between calculated CO₂ assimilation and measured CO₂ assimilation ranged between 0 and 9 μmol CO₂ cm⁻² s⁻¹.
10 μbar on average (Mott and O’Leary, 1984; Wong et al., 1985b; Parkhurst et al., 1988). Since CO₂ is being absorbed by cells throughout the leaf, these measurements are not simply related to conductance to diffusion across a leaf. A more direct method is to use an inert gas such as helium or N₂O. This has yielded values of intercellular conductance across the leaf of 0.33 mol m⁻² s⁻¹ for Xanthium (Farquhar and Raschke, 1978; Mott and O’Leary, 1984) and 0.017 or 0.07 mol m⁻² s⁻¹ for Zea mays (Long et al., 1989; Farquhar and Raschke, 1978, respectively). The lower value for Zea mays is probably associated with a very dense mesophyll layer through the center of leaves of some cultivars. As it is possible to determine intercellular conductance by this method only with amphistomatous leaves and since intercellular conductance to CO₂ diffusion involves lateral as well as vertical movement, these conductances are probably underestimates. Amphistomatous leaves with high rates of photosynthesis are probably unlikely candidates for a limiting conductance through intercellular airspace. It seems more likely that limiting intercellular airspace conductances would occur in leaves which have stomata only on one surface.

Another approach is to compare gas exchange in air with that in Helox (air in which nitrogen is replaced by helium) as rates of diffusion in Helox are 2.3 times faster than in air. Resistance to diffusion in intercellular airspaces can be reduced in Helox which could increase photosynthetic rate at a given substomatal CO₂ partial pressure. Helox increased photosynthetic rates by an average of 2 and 12% for amphistomatous and hypostomatous leaves, respectively (Parkhurst and Mott, 1990). Using the observed correlation between internal conductance and photosynthetic capacity shown in Fig. 10 (gᵢ = 0.012A) and taking values for the rate of CO₂ assimilation and pᵣ of 25 μmol m⁻² s⁻¹ and 249 μbar for amphistomatous and 9 μmol m⁻² s⁻¹ and 256 μbar for hypostomatous leaves, one can calculate that pᵣ was 166 and 173, respectively, for the two leaf types. Knowing that Helox increased the rates of CO₂ assimilation by 2 and 12% by decreasing intercellular airspace resistance by a factor of 2.3, one can calculate that intercellular airspace resistance in normal air represents about 10 and 57% of the internal resistance in amphistomatous and hypostomatous leaves, respectively.

A large intercellular airspace resistance was inferred for the thick Metrosideros leaves up an elevational transect (Vitousek et al., 1990; see Fig. 7G). A strong positive correlation was found between δ¹³C and leaf mass per unit leaf area (i.e. CO₂ drawdown from surrounding air to inside the chloroplasts increased with increasing LMA). From gas exchange measurements, they were able to show that the ratio of intercellular to ambient CO₂ partial pressures did not differ greatly along the elevational transect which left internal conductance as the most likely source of variation. Estimating gᵢ from the data presented of photosynthetic rate, pᵣ/pᵣ and Δ of leaf dry matter reveals a negative relationship between gᵢ and leaf mass per unit leaf area, LMA, (Fig. 11). The increase in LMA may reflect an increase in leaf thickness as well as an increase in tissue density, both of which are consistent with the calculated lowering of gᵢ. This trend is not evident in other species for which data is available. For tobacco, increased gᵢ was found in leaves that had been grown under higher irradiance which resulted in higher LMA (Evans et al., 1994). Syvertsen et al. (1995) pooled their species (peach, citrus and Macadamia) to obtain a negative dependence of gᵢ on LMA, but such a dependence is not evident when each species
is examined by itself. For both peach and citrus, growth in part shade resulted in lower LMA than for leaves in full sun as well as slightly lower $g_w$, although the differences were slight. Clearly more data is needed, especially for species which have dense or thick leaves. To resolve the separate contributions of intercellular and liquid components of the resistance pathway also calls for another approach, such as the combination of Helox with isotope discrimination or chlorophyll fluorescence. By examining $\Delta$ or chlorophyll fluorescence concurrently with gas exchange in both normal air and Helox, it would be possible to dissect the relative contribution of the two components. If intercellular airspaces constitute a significant resistance to $CO_2$ diffusion, then changing from air to Helox should result in a significant increase in $\Delta$ at a given $p/p_e$.

2. Intracellular Resistance

a) Surface Area of Chloroplasts Exposed to Intercellular Airspace, $S_c$

There is a striking analogy between $CO_2$ exchange across mesophyll cell walls in leaves and mammalian lungs. The resting exchange rate of $CO_2$ in mammals is 3 $\mu$mol (m alveolar surface)$^2$ s$^{-1}$, regardless of animal size over four orders of magnitude (Tenney and Remmers, 1963; Fig. 12). Wheat and tobacco photosynthesizing in sunlight have similar exchange rates per unit chloroplast surface area adjacent to intercellular airspace. Rapid exchange requires large surface areas to reduce gradients across the interfaces in both lungs and leaves. However, the similarity in $CO_2$ exchange rate per unit surface area is not due to similar conductances. In the lung, $CO_2$ partial pressure is, on average, 20 mbar while the partial pressure of $CO_2$ in the blood declines from 60 to 53 mbar as it passes through the lungs. The average partial pressure difference is thus 36 mbar. In wheat leaves, the difference from substomatal cavities to the sites of carboxylation is only 80 $\mu$bar. This suggests that the conductance to $CO_2$ from the air to the liquid phase is 450 times greater in leaves than in lungs. Allowing for the fact that $CO_2$ exchange rate increases ten-fold in an active mammal over the resting rate, the conductance in leaves is still about 45 times greater than in mammalian lungs. The reason for this difference is unclear given that the liquid pathlengths appear similar. Plants require a greater conductance to support the rapid exchange of $CO_2$ during photosynthesis, but at present we do not understand exactly how they achieve this.

For leaves where intercellular airspace resistance plays a minor role, Eq. (16) suggests that $g_w$ should be proportional to the surface area of chloroplasts exposed to intercellular airspace per unit leaf area, $S_c$ (Laish et al., 1970). Mesophyll structure of leaves is diverse, as shown in Fig. 7. Palisade tissue is found in many dicot leaves near the adaxial surface. This structure allows close packing of cells with a high surface to volume ratio while still leaving regular intercellular airspaces. The arrangement is interrupted beneath stomata to form sub-stomatal cavities, but generally stomatal density is less on adaxial than abaxial surfaces. Spongy mesophyll adjacent to abaxial surfaces allows free and unrestricted diffusion away from stomata on this surface.

Cell size or volume is related to nuclear DNA content or ploidy. Wheat provides a nice example of this as cell volume increases with ploidy (Dunstone and Evans, 1974). Mesophyll cells of diploid, tetraploid and hexaploid wheat had volumes of 6710, 13050 and 17860 $\mu$m$^3$, respectively (1:1.9:2.7, Kaminski, 1984). Along with larger cells, Rubisco content increases in proportion with ploidy level (wheat, Dean and Leech, 1982; alfalfa Molin et al., 1982) so that Rubisco per cell increases from 1:2:1:3:2 for diploid:tetraploid:hexaploid wheat (Pyke and Leech, 1987). If the cells were spherical, this would
result in a decline in the surface area to volume ratio relative to diploid cells by 20 and 28% for the tetraploid and hexaploid wheat, respectively. However, the cells become progressively more lobed with increasing ploidy (Dunstone and Evans, 1974, Chonan et al., 1977, Parker and Ford, 1982, Sasahara, 1982) such that exposed mesophyll cell surface per unit leaf area, \( S_{mes} \), is independent of ploidy (Kaminski et al., 1990). Mesophyll cell lobing was noted by Haberlandt (1914) to occur in many species and is illustrated in Fig. 13 for wheat (A,B,C), pine (E) and tobacco (F).

\( S_{mes} \) varies considerably between species and with growth irradiance. Turrell (1936) measured 11 species, finding \( S_{mes} \) ranged from 13.5 and 26.4 for shade and sun leaves of *Syringa vulgaris*, up to 62.5 for *Eucalyptus globulus*. While \( S_{mes} \) has been measured in quite a few studies, it is the proportion of \( S_{mes} \) that is covered by chloroplasts that is probably the more relevant measure that needs to be made. Interestingly, the proportion of cell wall covered by chloroplasts is not related to chloroplast number (Ellis and Leech, 1985). Mutants of *Ambidopsis* that have impaired chloroplast division have the same proportion of cell wall covered by chloroplasts as wildtype because the chloroplasts are larger (Pyke and Leech, 1992, 1994; Pyke et al., 1994). The proportion of cell wall covered varies between species, from 86–93% for tobacco (Evans et al., 1994), 76% for wheat (Evans, 1983a) and 73% for spinach (Honda et al., 1971) down to 26–34% for three shade species (Araus et al., 1986). Chloroplasts are generally adjacent to cell walls exposed to intercellular airspace in palisade tissue (Fig 13D; Psaras, 1986, Bolhar-Nordenkampf and Draxler, 1993, Psaras et al., 1996) as well as spongy tissue (Fig. 13F) where the absence of chloroplasts on walls shared by an adjacent cell is seen most readily.

The measurement of \( S_{e} \) is laborious and in the past it has been the total surface area of mesophyll cells exposed to airspace that has been measured (\( S_{mes} \), Nobel et al., 1975). Consequently there are not many data sets to generalize from yet. The available data is presented in Fig. 14. Setting aside *Macadamia*, *Kalanchoe* and aging wheat leaves, there is a remarkable similarity between all the remainder. Tobacco, recently fully expanded wheat flag leaves and sun- and shade-grown peach and citrus leaves, all share a conductance of 24 mmol (m chloroplast surface)\(^{-2}\) s\(^{-1}\) bar\(^{-1}\). This similarity is despite considerable variation in leaf thickness and porosity, again highlighting that the intercellular airspace is a less important determinant of \( g_{w} \). *Macadamia* has around half, and the thick CAM leaf of *Kalanchoe* around one quarter of the internal conductance of the other group, possibly indicating that these leaves do have a significant intercellular airspace resistance. The picture is further complicated by the change found in wheat leaves as they aged (Evans and Vellen, 1996). In the early stages of leaf aging, photosynthetic capacity and \( g_{w} \) were both declining in parallel without any change in exposed chloroplast surface area, presumably because the chloroplasts were becoming thinner. This suggests that the permeability of one or more component of the liquid diffusion pathway declined with increasing age. At present this is an intractable problem experimentally.

Haberlandt (1914, Fig. 107) observed ‘in the photosynthetic tissues of higher plants, the chloroplasts adhere exclusively, or in great part, to those walls which abut upon airspaces; by this means they evidently obtain the most favorable conditions for the absorption of carbon dioxide.’ The reason for this is that CO\(_{2}\) diffuses 10,000 times more slowly in water than air so that short liquid pathways are essential if rapid CO\(_{2}\) exchange is to occur. The other way to reduce resistance is to increase the surface area available for gas exchange. Both are employed by the leaf. Photosynthetic capacity of a leaf can be increased in many species by higher growth irradiance. Increases in photosynthetic capacity require extra Rubisco and thylakoid proteins so that the chloroplast volume per unit leaf area increases. At the same time, the internal leaf surface increases so that changes in the ratio of photosynthetic capacity to chloroplast surface area exposed to intercellular airspace are small (Nobel et al., 1975; Evans et al., 1994).

### b) Liquid Phase Resistance

The resistances imposed by the cell wall and segments of the liquid phase can be roughly calculated. This requires assumptions about the effective diffusivities of CO\(_{2}\) through the cell wall and cytosol and the permeabilities of the plasmalemma and chloroplast envelope (Evans et al., 1994). The resistance to CO\(_{2}\) imposed by plant membranes is unknown. Measurements have been made on red blood cells (167 s m\(^{-1}\), 4.17 m\(^{2}\) s bar mol\(^{-1}\); Solomon, 1974), lecithin-cholesterol lipid bilayers (286 s m\(^{-1}\); Gutknecht et al., 1977) and the plasma membrane of *Chlamydomonas*...
Fig. 13. Cell lobing and chloroplast positioning. In order to maximize surface area to cell volume, mesophyll cells of many species are lobed. Wheat (A, B, C longitudinal), tobacco (D, F paradermal) and Pinus (E). Chloroplasts are appressed against the cell surface adjacent to airspace and rarely against walls with neighboring cells. When viewed in cross-section, wheat mesophyll cells appear circular or elliptical. When viewed in longitudinal section, the lobing becomes apparent, although depending on where the section is cut, the lobes may appear like small circular cells.
This last resistance value is so high that photosynthesis in higher plants would not be possible without an active concentrating mechanism whereas the first two estimates result in about 50% of the liquid resistance being imposed by the lipid membranes. Once inside the chloroplast, diffusion across the chloroplast is facilitated by carbonic anhydrase, CA, which rapidly interconverts CO$_2$ and bicarbonate so that many more molecules are available for diffusion (at pH 8, the ratio of bicarbonate to CO$_2$ is 45). For tobacco, where intercellular airspace resistance is probably negligible, the internal resistance is 43 m$^2$ chloroplast s bar mol$^{-1}$ (Evans et al., 1994). By subtracting from this the estimated resistances imposed by the cell wall (12.5), membranes (3 x 4.17) and cytosol (2.5), we are left with 16 m$^2$ chloroplast s bar mol$^{-1}$ (38%) due to the resistance within the chloroplast.

It has been possible to reduce CA activity to 1% of wild-type activity in transgenic tobacco containing an antisense gene for CA which resulted in lowering the CO$_2$ partial pressure in the chloroplast by about 15 μbar and decreasing the internal conductance by 25–30% (Price et al., 1994; Williams et al., 1996). Another way to examine the role of CA has been to inhibit the activity with ethoxyzolamide (Peltier et al., 1995). Working with Commelina communis, Peltier et al. found that 97% inhibition of CA activity reduced the CO$_2$ diffusion constant by 20%. While the authors stated that the ratio of $p_c/p_a$ was not significantly different and therefore that the major part of CA was not involved in the transfer of CO$_2$, one can calculate that internal conductance was actually reduced by nearly 20%, similar to the reduction in the CO$_2$ diffusion constant. Thus independent techniques and laboratories have all observed a similar effect following removal of 95% of CA activity, confirming that CA facilitates CO$_2$ diffusion across the chloroplast by reducing diffusion resistance within the chloroplast. CA plays a similar role in facilitating CO$_2$ diffusion and interconversion in red blood cells. Surprisingly, complete inhibition of CA in the blood by specific inhibitors did not noticeably affect CO$_2$ transport in the bloodstream (Schmidt-Nielsen, 1991).

In a study of transgenic tobacco having excess phytochrome, Sharkey et al. (1991) observed that the transgenic plants had a lower photosynthetic capacity than expected on the basis of their Rubisco content which was actually greater than the wild-type plants. The leaves were thicker and contained cup-shaped chloroplasts which, it was suggested, reduced the internal conductance because of the additional thickness of cytoplasm that needed to traverse to reach the sites of carboxylation. Detailed measurements revealed that the average thickness of the cytoplasm at the middle of the chloroplast was increased from 0.25 to 0.55 μm in the leaves of the transgenic plants. This increase in cytoplasmic thickness seems insufficient to explain the lower photosynthetic capacity, but reflects the uncertainties in our current understanding. Although carbonic anhydrase is mainly located in chloroplasts, significant amounts (13%) have also been found in the cytoplasm of Solanum tuberosum (Rumeau et al., 1996) and 1 to 3% of CA in spinach and wheat has been localized to the plasmalemma (Utsunomiya and Muto, 1993). It is therefore possible that CA could be increasing the permeability of CO$_2$ through the plasmalemma and cytoplasm as well as the chloroplast stroma. Facilitation of CO$_2$ diffusion by CA in the cytoplasm would be less effective because the lower cytoplasmic pH results in a smaller ratio of bicarbonate to CO$_2$ than in the chloroplast.
photosynthesis requires a specialized leaf anatomy where photosynthetic cells are organized in two concentric cylinders (Fig. 15). The ‘Kranz’ or wreath anatomy was recognized by Haberlandt (1914, Fig. 113) who speculated at the time ‘It is uncertain whether this green inner sheath merely represents an unimportant addition to the chlorophyll-apparatus of the plant, or whether there exists some as yet undiscovered division of labor between the chloroplasts in the sheath and those in the girdle-cells’. The $C_4$ photosynthetic pathway is characterized by a $CO_2$ concentrating mechanism that involves the coordinated functioning of mesophyll and bundle-sheath cells within a leaf. $CO_2$ is initially assimilated into $C_4$ acids by phosphoenol pyruvate (PEP) carboxylase in mesophyll cells. These acids then diffuse to the bundle-sheath cells where they are decarboxylated. This concentrates $CO_2$ in the bundle-sheath which enhances RuBP carboxylation while inhibiting RuBP oxygenation (Hatch and Osmond, 1976). Thin-walled
mesophyll cells adjacent to intercellular airspace radiate from thick-walled bundle-sheath cells. The diffusion of CO\textsubscript{2} back out from the bundlesheath limits the efficiency of the CO\textsubscript{2} concentrating mechanism and many attempts have been made to quantify the CO\textsubscript{2} diffusion resistance across the bundlesheath (Jenkins et al., 1989; Brown and Byrd, 1993; Hatch et al., 1995). Limitations to CO\textsubscript{2} diffusion from intercellular airspaces to the mesophyll cells have received less attention (Longstreth et al., 1980).

### B. From Intercellular Airspace to the Mesophyll

The necessity for metabolite transport between mesophyll and bundle-sheath cells requires intimate contact between these cells and limits the amount of mesophyll tissue which can be functionally associated with bundlesheath tissue. For example, the number of chlorenchymatous mesophyll cells between adjacent bundlesheaths is usually between two and four. Mesophyll surface area exposed to intercellular airspace per unit leaf area is slightly less in C\textsubscript{4} than in C\textsubscript{3} species (Longstreth et al., 1980; Pearcy et al., 1982, Dengler et al., 1994) and thus the surface area available for CO\textsubscript{2} diffusion is also less.

No robust techniques are presently available that allow the estimation of conductance to CO\textsubscript{2} diffusion from intercellular airspace to sites of PEP carboxylation. Carbon isotope discrimination cannot be used because of the low discrimination factor of PEP carboxylase and the confounding effects of CO\textsubscript{2} leakage from the bundlesheath (Henderson et al., 1992). Similarly, chlorophyll fluorescence signals are not suitable as bundlesheath and mesophyll cells have different chloroplast populations. In an approach analogous to Evans (1983b), Pfeffer and Peisker (1995) compared the change in the reciprocal of initial slope of a CO\textsubscript{2} response curve against PEP carboxylase activity for maize leaves grown under natural light or shade. By assuming that internal conductance was the same for all leaves, they derived an estimate of 0.8 mol m\textsuperscript{-2} s\textsuperscript{-1} for maize (Pfeffer and Peisker, 1995). The value obtained for wheat was 0.49 mol m\textsuperscript{-2} s\textsuperscript{-1} bar\textsuperscript{-1} (Evans, 1983b) which is similar to the value obtained by the more rigorous stable carbon isotope method (von Caemmerer and Evans, 1991). Thus, on this limited evidence, maize internal conductance is greater than that of wheat.

For both C\textsubscript{3} and C\textsubscript{4} species one can calculate a minimum internal conductance (Evans and von Caemmerer, 1996). This limit is reached when \( p_c \) is reduced to the CO\textsubscript{2} compensation point, \( \Gamma \), \( \left( g_{\text{min}} = \frac{A}{(p_c - \Gamma)} \right) \). At 25 °C, high light and ambient CO\textsubscript{2}, intercellular CO\textsubscript{2} is \( \approx 100 \) \( \mu \text{bar} \) in C\textsubscript{4} species versus 250 \( \mu \text{bar} \) for C\textsubscript{3} species and the compensation point is close to zero in C\textsubscript{4} versus \( \approx 50 \) \( \mu \text{bar} \) in C\textsubscript{3} species. Thus for the same CO\textsubscript{2} assimilation rate, \( g_{\text{min}} \) of C\textsubscript{3} species needs to be approximately twice that of C\textsubscript{3} species. Given that the exposed mesophyll surface is less in C\textsubscript{4}, this requires conductance across the cell wall through to the cytosol in C\textsubscript{4} species to be more than double that in C\textsubscript{3} species. There are several possible contributing factors. Firstly, mesophyll cell walls of C\textsubscript{4} species may be thinner than for C\textsubscript{3} species (0.07 \( \mu \text{m} \) Amaranthus retroflexus, Longstreth et al., 1980; cf 0.3 \( \mu \text{m} \) Nicotiana tabacum, Evans et al., 1994) although a general survey is needed to confirm this. Secondly, in contrast to C\textsubscript{3} plants where CA is mainly located in the chloroplast to facilitate CO\textsubscript{2} diffusion, in C\textsubscript{4} plants CA is found in the cytosol alongside PEP carboxylase (Hatch and Burnell, 1990). Utsunomiya and Muto (1993) separated a plasmalemma fraction from a leaf extract and found that it contained 20–60% of CA activity in Zea mays leaves compared to 1–3% in wheat. CA in the plasmalemma may facilitate CO\textsubscript{2} movement across membranes, perhaps even delivering HCO\textsubscript{3} into the cytosol. Thirdly, the initial carboxylation reaction by PEP carboxylase which utilizes HCO\textsubscript{3} occurs in the cytosol, so the liquid diffusion path in C\textsubscript{4} plants may be considerably shorter and does not have to cross the chloroplast envelope. It is likely that CO\textsubscript{2} assimilation rate will be sensitive to reduction in CA activity in C\textsubscript{4} plants because PEP carboxylase would have to rely on the uncatalyzed rate of conversion of CO\textsubscript{2} to bicarbonate which is 10,000 times slower than the catalyzed rate. If the observed internal conductance of C\textsubscript{3} leaves (Fig. 10) was also assumed for C\textsubscript{4} leaves, this would result in values for the CO\textsubscript{2} partial pressure in C\textsubscript{4} mesophyll cytoplasm of only 17 \( \mu \text{bar} \) compared to 167 \( \mu \text{bar} \) in the chloroplasts of C\textsubscript{3} leaves.

### C. Across the Bundlesheath

A low conductance to CO\textsubscript{2} diffusion across the bundlesheath is an essential feature of the C\textsubscript{4} pathway. It effectively limits CO\textsubscript{2} exchange with the atmosphere and CO\textsubscript{2} acid decarboxylation is the major source of CO\textsubscript{2} in this compartment. Inhibiting PEP carboxylase activity reduced CO\textsubscript{2} assimilation rate by 80–98% (Jenkins, 1989). The conductance to CO\textsubscript{2} diffusion
across bundle sheath walls is considerably less than across mesophyll cell walls, with estimates ranging from 0.6–2.4 mmol m⁻² leaf s⁻¹ or 0.5–0.9 mmol (m bundle sheath)⁻² s⁻¹ fordifferent C₄ species (Jenkins et al., 1989; Brown and Byrd, 1993; Brown, 1997) compared to 25 mmol m⁻² chloroplast s⁻¹ for tobacco (Evans et al., 1994). The absence of CA in the bundlesheath prevents the rapid conversion of CO₂ to bicarbonate which would increase the diffusion of CO₂ out of the bundle sheath, similar to the way it facilitates CO₂ diffusion across chloroplasts in C₃ plants. When CA from tobacco was expressed in transgenic Flaveria bidentis leaves, leakage of bicarbonate out of the bundle sheath was enhanced, as measured by concurrent carbon isotope discrimination and gas exchange measurements (von Caemmerer et al., 1997). The liquid pathlength is also long, imposing a considerable resistance. For NAD ME decarboxylation types, chloroplasts are centripetally arranged (Fig. 15 A,B) and decarboxylation occurs in the mitochondria that are interposed between the chloroplasts (Hatch et al., 1975, Brown and Hattersley, 1989). In the NADP ME type, decarboxylation occurs in the bundle sheath chloroplasts which are either located centrifugally in the monocots (Zea Fig. 15C) or centripetally in the dicots (Gomphrena Fig. 15D). Furthermore, in C₄ species that have either centrifugally arranged chloroplasts (eg Fig. 15C,F) or bundlesheaths with uneven cell outlines, the bundle sheath cell wall is lined by a suberised lamella, which may also help reduce conductance to CO₂. The PKC type has decarboxylation in the cytosol, so it makes sense for chloroplasts to be centrifugally located to catch the CO₂ as it diffuses away from the vascular strand (Urochloa Fig. 15F).

The C₄ cycle consumes energy and so leakage of CO₂ from the bundle sheath is an energy cost to the leaf which represents a compromise between keeping CO₂ in, letting O₂ out and letting metabolites diffuse in and out at rates fast enough for the rate of CO₂ fixation. The leakage depends upon the balance between the rates of PEP carboxylation and Rubisco activity and the conductance of the bundle sheath to CO₂. In transgenic Flaveria bidentis leaves where antisense reduction in Rubisco content altered the balance between the C₄ and C₃ cycles, leakiness increased from 24% in wild-type to 37% (von Caemmerer et al., 1997). Various estimates have been made of what proportion of CO₂ fixed by PEP carboxylase subsequently leaks out of the bundle sheath. This has been termed leakiness, and estimates have ranged from 8% to 50% (see Henderson et al., 1992; Hatch et al., 1995). Meinzer and Zhu (1998) found variation in dry matter Δ correlated with variation in quantum yield associated with both leaf nitrogen content and genotype in Saccharum species and suggested that this was most likely due to variation in leakiness. Leaves with higher N contents had an increased ratio of Rubisco:PEP carboxylase activity and lower calculated leakiness. Despite this and earlier suggestions that leakiness differed between C₄ decarboxylation types (being less in species with a suberised lamella, Hattersley, 1982), recent measurements of leakiness have found little variation (Henderson et al., 1992; 8–12% Hatch et al., 1995). Extensive concurrent measurements of Δ with gas exchange have revealed little variation in leakiness either between species, or with variation in photosynthetic rate due to short-term changes in CO₂, irradiance and temperature, or with longer term changes in leaf nitrogen content (average 21%, Henderson et al., 1992, 1994). Since leakiness is determined not only by the physical conductance of the bundle sheath, but also by the balance of the capacities of the C₄ and C₃ cycles, this suggests that the biochemistry of C₄ photosynthesis is highly regulated.

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Carbonic anhydrase, an enzyme which catalyzes the reversible hydration of $\text{CO}_2$, is a major protein component of most photosynthetic microorganisms and higher plant tissues. Once thought to be represented in plants by a single enzyme type, it is now apparent that DNA sequences and/or the encoded proteins for the evolutionarily distinct $\alpha$, $\beta$, and $\gamma$ forms of carbonic anhydrase are present in cyanobacteria, green algae, and higher plants. While exhibiting a wide range in structure, localization, and regulation of expression, some progress has been made in the establishment of roles for these various enzyme forms. It would appear that the primary role of many of the $\alpha$ and $\beta$ isoforms is the establishment of inorganic carbon species equilibration. As a result of this activity, enzymes or transport systems which require either $\text{CO}_2$ or $\text{HCO}_3^-$ are not limited by the slow, uncatalyzed rate of $\text{CO}_2/\text{HCO}_3^-$ interconversion. In contrast, little is known about patterns of expression or role(s) for $\gamma$ isoforms. Recent studies on carbonic anhydrase described in this chapter include the isolation and characterization of new isoforms, the generation and phenotypic description of carbonic anhydrase mutants, as well as elucidation of mechanisms responsible for regulation of expression.
I. Introduction

Carbonic anhydrase (CA; EC 4.2.2.1) is a zinc-containing enzyme which catalyzes the interconversion of CO₂ and HCO₃⁻. In this reversible hydration/dehydration reaction, described as CO₂ + H₂O ⇌ HCO₃⁻ + H⁺, the final equilibrium concentrations of HCO₃⁻ and CO₂ are established primarily by the pH of the aqueous environment. For example, when inorganic carbon species are at equilibrium and a pH value of 8.0, over 80% of the inorganic carbon (Cᵢ) in solution is in the form of HCO₃⁻. Conversely, as the pH declines the proportion of Cᵢ in solution in the form of CO₂ increases, such that at pH 6.0, approximately 75% of the total Cᵢ is CO₂. Why the need for carbonic anhydrase in biological systems if the interconversion of CO₂ and HCO₃⁻ will proceed spontaneously? The answer is readily apparent if one examines the uncatalyzed rate of Cᵢ species equilibration. In the absence of the enzyme, the establishment of CO₂ and HCO₃⁻ equilibrium is very slow, exhibiting a t½ of approximately 15 s at 25 °C and pH 8.0. As such, enzymatic reactions or active transport processes that utilize a specific Cᵢ species preferentially could soon experience declines in activity if the rate of substrate use exceeds the rate of uncatalyzed Cᵢ equilibration. Reductions in activity would become more extreme as the HCO₃⁻/CO₂ ratio moves further away from equilibrium. Where CA is present, the rapidity of the enzyme catalyzed reaction effectively eliminates the possibility of Cᵢ equilibration limiting a biological process. As CA appears to be found in all organisms yet examined, the ubiquitous nature of this enzyme would seem to indicate an important role in many physiological events. Indeed, in animal systems CA has a pivotal position in such diverse processes as respiration, kidney function, and bone formation (Tashian, 1989). The abundance of CA in many photosynthetic organisms, the molecules involved in the catalyzed reaction (CO₂, HCO₃⁻ and H⁺), and its primary spatial and temporal association with photosynthetic tissue, have all suggested to many researchers that CA should also play a key role in photosynthesis. This speculation generated much research activity in the past however only recently has more direct evidence for a specific role of CA in some photosynthetic organisms been shown, and indeed, much more work needs to be done. In the following sections I will attempt to highlight some of the recent progress which has been made on characterization of the enzyme and determination of role(s) of CA in higher plants, microalgae, and cyanobacteria. In addition, I will also suggest some important areas for future research. I have not attempted to review all the literature in this area as it is voluminous and there are many older reviews on plant and algal carbonic anhydrases that provide a historical perspective (Poincelot, 1979; Reed and Graham, 1981; Graham et al., 1984; Aizawa and Miyachi, 1986; Tsuzuki and Miyachi, 1989). For complementary information, readers are also directed to two comprehensive reviews on CA in plants and other photosynthetic organisms by Sultemeyer et al. (1993) and Badger and Price (1994).

II. Enzyme Types, Structures And Kinetics

Carbonic anhydrases from a variety of sources, although catalyzing the same reaction, have been recently sub-divided on the basis of primary amino acid sequence into three major groups (Hewett-Emmett and Tashian, 1996). These groups are (a) α-CAs, typified by the well studied vertebrate-type enzyme (Tashian, 1989); (b) β-CAs, the group first characterized as a higher plant, chloroplast-localized enzyme (Burnell et al., 1990a; Fawcett et al., 1990; Roeske and Ogren, 1990; Majeau and Coleman, 1991, 1992); and (c) γ-CAs, a recently identified group first characterized in the Archaeabacteria (Alber and Ferry, 1994). The highly divergent sequences of these three groups certainly suggests multiple evolutionary origins but what is more intriguing is that many single organisms appear to express CAs from more than one group. For example, the cyanobacterium Synechococcus PCC 7942 expresses both α- and β-CAs and also contains a protein with significant amino acid sequence homology to the γ group of CAs (Fukuzawa et al., 1992; Yu et al., 1992; Price et al., 1993; Soltes-Rak et al., 1997). The higher plant Arabidopsis also contains DNA sequences significantly similar to α- and γ-CAs as well as the expressed β-CA isoforms found in the chloroplast and cytosol (Raines et al., 1992; Fett and Coleman, 1994; Newman et al., 1994). Although regulation of expression and localization studies for the ‘non-plant-like,’ α- and γ-CAs in higher plants has not been done, characterization of these enzymes...
types in cyanobacteria and eukaryotic algae suggest that higher plants will also be found to use all three enzyme groups for specific functions in various tissues, cells and/or organelles.

Although all three CA groups are zinc-metalloenzymes, with one Zn atom localized in the active site of each monomer, there are considerable differences in the structures of the active sites. For the α-type enzyme isolated from humans and animals, many crystal structures have shown that three essential His residues co-ordinate the position of the Zn atom in the active site at the bottom of a conical cavity (Tashian, 1989; Silverman, 1991). The sequence similarity between the periplasm-localized CAH1 and CAH2 proteins isolated from the eukaryotic green alga Chlamydomonas reinhardtii (Fujikawa et al., 1990) and the similarly localized ECA proteins in the cyanobacteria (Soltes-Rak et al., 1997) suggest that these α-type CAs will also have homologous active sites. The reaction mechanism within the active site of mammalian α-type enzymes involves a zinc-bound water molecule which can ionize to a Zn-bound hydroxide. In this basic form of the enzyme the CO₂ hydration reaction is catalyzed whereas the dehydration of HCO₃⁻ requires the protonated enzyme form. The actual catalytic mechanism is a two step process (Silverman, 1991):

\[
\text{E-Zn-OH}^- + \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{E-Zn-H}_2\text{O} + \text{HCO}_3^- \quad (1)
\]

\[
\text{E-Zn-H}_2\text{O} + \text{buffer} \leftrightarrow \text{E-ZnOH}^- + \text{buffer-H}^+ \quad (2)
\]

Equation (1) describes the interaction of the catalytically Zn-bound hydroxide with CO₂, the formation of HCO₃⁻ and the replacement of the OH⁻ with a H₂O bound to the Zn. Equation (2) describes what is the rate-limiting step in the reaction, the regeneration of the Zn-bound hydroxide by the protolysis of the Zn-bound H₂O and the transfer of the liberated H⁺ to the surrounding medium (identified as a buffer).

A. α-Carbonic Anhydrase Enzymes

In terms of structure, the best studied examples of an α-CA from a photosynthetic organism are the homologous periplasmic proteins encoded by cah₁ and cah₂ in the eukaryotic alga Chlamydomonas. Translated as a 42–44 kDa precursor protein on 80S ribosomes, this extracellular CA is proteolytically processed to yield 35 and 4 kDa monomers (Coleman and Grossman, 1984; Kamo et al., 1990). The holoenzyme is approximately 76 kDa and is tetramer of two 35 kDa monomers linked by a disulfide bond, and two 4 kDa monomers each linked to the larger subunit by a disulfide bond (Kamo et al., 1990). Following synthesis, the protein is glycosylated, assembled and exported in the periplasmic space. Amino acid sequence comparisons with vertebrate α-CAs indicates about 20% overall sequence similarity, however extensive conservation of zinc-binding His residues and other important active site amino acids certainly suggests a common catalytic mechanism. Recently, an extracellular α-CA has been identified in the cyanobacteria Synechococcus and Anabaena (Soltes-Rak et al., 1997). Again strict conservation of almost all active site residues suggest a common catalytic mechanism however the oligomeric structure of this enzyme has yet to be determined.

B. β-Carbonic Anhydrase Enzymes

Although no crystal structures are yet available for the β-type CAs, site-directed mutagenesis studies and extended X-ray fine structure analysis indicate that two Cys and one His residue are involved in coordination of the Zn atom in the active site (Provart et al., 1993; Bracey et al., 1994). Specific site-directed changes of H220N, C160S, or C223S in the gene encoding chloroplastic localized CA isolated from pea (Pisum sativum) (Provart et al., 1993) and the corresponding changes H210Q, C150A, or C213A in a similar cDNA encoding spinach (Spinacea oleracea) chloroplast CA (Bracey et al., 1994) all resulted in an inactive enzyme which was incapable of binding Zn. Additional site-directed changes in the pea enzyme of H209N and E276A produced enzymes with compromised catalytic activity which could be restored by the addition of imidazole buffers capable of entering the active site. Similar lesions and imidazole buffer complementation in the α-type mammalian CAII enzymes involving H64 suggest that this residue is involved in the intramolecular transfer of protons necessary for regeneration of the catalytically active Zn-hydroxide. The pea and spinach enzyme data suggest that a similar strategy may be employed by the β-type plant enzymes. Kinetic analysis of pea (Johansson and Forsman, 1993) and spinach (Rowlett et al., 1994) show clearly that although structurally very different, β-type CAs isolated from higher plants are as efficient as the well
described α-type mammalian CAII enzyme. For example, in a recent, detailed kinetic study of the chloroplastic localized pea CA, a turnover number/subunit (kcat) at pH 9.0 of $4 \times 10^4 \text{s}^{-1}$ and a $k_{cat}/K_m$ value of $1.8 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ were obtained. Representative values for the human CAII are $1 \times 10^6 \text{s}^{-1}$ and $1.2 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ (Johansson and Forsman, 1993). Kinetic analysis of the spinach enzyme have provided similar data and also show that a $\text{H}^+$ transfer step is the rate determining factor in the catalytic mechanism, although it was not possible to conclude if an intramolecular $\text{H}^+$ transfer system was operational (Rowlett et al., 1994). Taken together, the structural and kinetic data indicate that the β-type enzyme active site residues and organization are different from the α-type enzymes but that the catalytic mechanisms are similar.

The oligomeric structure of the β-type enzyme in the absence of crystal structures is still unresolved and the literature contains a number of reports describing native enzymes of differing molecular mass. The most recent estimates however, indicate a structure of eight monomeric units per oligomer for CAs isolated from leaves of dicots. (Guliev et al., 1992; Rumeau et al., 1996; Bjorkbacka et al., 1997). Cysteine residues have also been implicated in interaction of subunits, and a model of the chick pea leaf enzyme based on electron microscopy suggests a P 422 symmetry for the octomer (Guliev et al., 1992; Bjorkbacka et al., 1997). Reported monomeric sizes of leaf enzymes range from 24 kDa (Pisum) to 31 and 35 kDa (Flavaria) indicating some heterogeneity which may reflect differences between cytosolic and chloroplastic leaf isoforms and/or variation in transit peptide processing sites following uptake of the nascent polypeptide into the chloroplast (Johansson and Forsman, 1992; Forsman and Pilon, 1995; Ludwig and Burnell, 1995). Few studies of monocot leaf CAs have been performed, however older data suggest that the oligomeric form may be a dimer (Reed and Graham, 1981). Recently, isolation of cDNAs encoding chloroplast-localized CAs from barley (Bracey and Bartlett, 1995) and rice (Suzuki and Burnell, 1995) have shown that the monomeric sizes and overall aa sequences are similar to CAs from leaves of dicots although a 10 aa C terminal sequence conserved among dicot CAs is missing in monocots. Sufficient aa sequence variation does exist, however, such that antibodies directed against spinach CA (a dicot) fail to cross-react with CAs from C₃ and C₄ monocots (Okabe et al., 1984, Burnell, 1990) whereas antisera derived against maize leaf CAs exhibited some cross-reactivity with both C₃ and C₄ monocots and dicots (Burnell, 1990). A recent paper by Burnell and Ludwig (1997) describes the isolation and characterization of two cDNAs encoding mesophyll cell CA isoforms. Although the cDNAs have open reading frames of 71 and 53 kDa, immunological evidence shows that the two 180 kDa oligomeric native structures are composed of 27 kDa or 45–47 kDa monomers, suggesting some form of translational or post-translational processing. Certainly more detailed analysis of the oligomeric structure of monocot CAs is necessary before definitive statements on differences with dicots oligomeric structures can be made.

C. γ-Carbonic Anhydrase Enzymes

The only characterized example of a γ-CA is from the methanogenic archaeabacterium Methanosarcina thermophila (Alber and Ferry, 1994) although similar deduced amino acid sequences have been found in photosynthetic organisms such as the CcmM protein in Synechococcus which is involved in the CO₂ concentrating mechanism (Price et al., 1993), and other deduced amino acid sequences from cDNAs obtained from Arabidopsis (Newman et al., 1994). With no aa sequence similarity to the other CA groups, the holoenzyme in Methanosarcina appears to be homotrimer of the 37 kDa monomer. Interestingly, crystal structure analysis has shown the coordination of the Zn atoms (three per holoenzyme) is a function of His residues from two adjacent monomers resulting in the three active sites at the monomer interfaces (Kisker et al., 1996). The geometry of the His residues required for Zn binding is similar to that seen in α-CAs suggesting a similar catalytic mechanism.

III. Localization, Regulation of Expression and Role

A. C₄ Plants

It is now generally accepted that the principal enzyme in the photosynthetic tissue of C₄ plants is a β-CA localized in the cytosol of mesophyll cells (Hatch and Burnell, 1990). Little if any CA activity is found in bundle-sheath cells. To date the best data sets were obtained by separation of the two cell types and
estimating levels of mesophyll cell contamination of bundle sheath preparation using mesophyll enzyme markers such as PEPCase and pyruvate, $P_i$ dikinase (Burnell and Hatch, 1988). These studies show that CA activities of bundle sheath cells rarely exceed the level of contaminating mesophyll enzyme activities, suggesting little if any expression of CA outside of the mesophyll cell environment. Although most of the CA in the mesophyll tissue appears to be soluble and cytoplasmic, there is at least one study indicating that a portion of the CA, as determined by activity and western blot analysis, is associated with the cytoplasmic side of purified plasma membranes from Zea mays (Utsunomiya and Muto, 1993). It has been suggested that two CA cDNAs recently isolated from Zea encode the plasma membrane associated form and a soluble cytosolic enzyme (Burnell and Ludwig, 1997). Questions on CA localization in C$_4$ plants could benefit from careful cytological studies involving in situ hybridization of both protein antibody and RNA probes.

Models of C$_4$ photosynthesis and hypothesized roles for CA predict the observed pattern of localization. Within the mesophyll cells, CA catalyzes the hydration of CO$_2$, which diffuses across the plasma membrane. The resulting HCO$_3^-$ is then fixed by PEPCase, thus initiating the C$_4$ pathway. Quantitative analysis of CA activity in Zea also support this role. By taking into account estimated mesophyll intracellular CO$_2$ concentrations of 4 $\mu$M and CA $K_m$ CO$_2$ and V$_{max}$ values of 2 mM and 34,000 $\mu$mol CO$_2$ hydrated min$^{-1}$, mg Chl$^{-1}$, respectively, Burnell and Hatch (1990) determined that there was just sufficient CA activity in vivo to support observed rates of C$_4$ photosynthesis. What is particularly noteworthy of this study is that it addresses the supposed excess of CA activity long commented on by many researchers. By estimating in vivo activities in terms of mol CO$_2$ hydration capacity instead of the arbitrary and physiologically irrelevant Wilbur-Anderson units (determined at saturating levels of CO$_2$), the measured CA levels are now seen to be necessary component of the C$_4$ pathway. In an additional point on the role of CA in the C$_4$ mesophyll cell, calculations of CO$_2$ conductance across the cell wall to the cytoplasm indicate that the flux appear to be approximately two-fold higher than in C$_3$ plants (Evans and von Caemmerer, 1996). Although a number of possible factors, including thinner cell walls, and shorter diffusion paths may contribute to more rapid CO$_2$ flux, as reported in Zea mays it is possible that the significant amounts of CA activity associated with the mesophyll plasmalemma may facilitate CO$_2$ diffusion into the mesophyll cell (Utsunomiya and Muto, 1993).

Inhibition of CA activity in C$_4$ plants following vacuum infiltration of leaf pieces with the sulfonamide inhibitor ethoxyzolamide has also shown that CA plays a prominent role in photosynthesis. At low levels of external C$_i$ inhibition of photosynthetic O$_2$ evolution approached 80 to 90% when leaf pieces of Zea and Amaranthus were treated with the CA inhibitor (Badger and Pfanz, 1995). The degree of inhibition was reduced as external C$_i$ concentrations were increased. A somewhat puzzling observation in these studies however was that even when treated with ethoxyzolamide, rates of photosynthesis still exceeded the uncatalyzed rate of CO$_2$ production in the external medium. A number of explanations were provided. It was possible that inhibition of all cytosolic CA activity was not achieved, or that the high external HCO$_3^-$ concentration may access the mesophyll cells directly through the apoplasm and plasmodesmata. It was also proposed that an additional CA (perhaps the reported plasma membrane-localized enzyme) was less sensitive to ethoxyzolamide inhibition and was still able to facilitate CO$_2$ uptake and intracellular hydration. Whatever the explanation, the effective inhibition of photosynthesis by the reduction of CA activity, particularly at low external C$_i$ concentrations does support the importance of CA in the C$_4$ pathway.

That CA activity is a requirement for initiation of the C$_4$ pathway is also supported by data on regulation of expression. In Zea, exposure to light and increased N availability result in enhanced levels of CA and PEPCase activity (Burnell et al., 1990b). These changes in activity resulted from co-ordinated increased transcript abundance and not from light activation of the enzymes or other post-translational events. Experiments with various N sources and the glutamine synthetase inhibitor, methionine sulfoximine (MSX), have shown that N stimulation of CA and PEPCase expression appears to be mediated by changes in Gln levels (Sugiharto et al., 1992). This amino acid and/or its downstream metabolites seem to act as a positive effector resulting in a significant increase in CA and PEPCase transcript levels (Sugiharto et al., 1992). The similarity in patterns of regulation of expression of CA and PEPCase by light and N suggest that common mechanisms of gene activation are present. It would be interesting to determine if similar regulatory cis elements are
present in genomic promoter sequences of both CA and \text{C}_4 \text{PEPCase} isoforms.

A similar strategy of juxtaposing empirical data and modeling was employed to show that a bundle-sheath cell localized CA would be an impediment to the efficient Rubisco catalyzed fixation of CO$_2$ generated by the decarboxylation of \text{C}_4 acids. All decarboxylases in the bundle sheath cells generate CO$_2$ as a product (Jenkins et al., 1987). As such, and as a result of the slow rate of uncatalyzed hydration, the intracellular level of CO$_2$ in this tissue is estimated to be at least 70 \text{ \mu M}, significantly above equilibrium levels (Jenkins et al., 1989). As CO$_2$ is the active species for Rubisco, the carboxylation reaction is favored and little if any oxygenase activity occurs. If CA were present, the catalyzed conversion of CO$_2$ to HCO$_3^-$ would rapidly proceed to chemical equilibrium with the concomitant reduction in the rate of carboxylation. In addition, the formation of HCO$_3^-$ would result in an increased C$_i$ leak rate from the bundle sheath cells down a concentration gradient through the plasmodesmata and into the mesophyll cells. Modeling the impact of CA on bundle sheath cell CO$_2$ concentrations indicates that maintenance of CO$_2$ concentrations in excess of 100 \text{ \mu M} cannot occur if the hydration rate exceeds approximately 50 times the uncatalyzed rate (Burnell and Hatch, 1988). This level of CA activity represents less than 5\% of the average total CA activity in \text{C}_4 plant leaves. Measured CA levels appear to be even lower (Burnell and Hatch, 1988).

The use of \text{C}_4 transgenics would provide some interesting data on the role of CA in these plants. Recently, significant advances have been made in this area. Antisense technology has been used to assess the impact of reduced Rubisco activity in the \text{C}_4 plant \textit{Flaveria bidentis} (Furbank et al., 1996). Using a similar strategy, mesophyll cell-specific antisense expression directed against the cytoplasm-localized CA should result in a severe inhibition of photosynthetic performance, particularly in a low external CO$_2$, high light environment. Additionally, the over-expression of CA activity in bundle sheath tissue should also result in a inhibition of photosynthesis by enhancing the C$_i$ leak rate from these cells. This has now been shown experimentally. Using the same host and transformation strategy, tobacco CA over-expression in leaves of \textit{Flaveria} resulted in plants with up to a 25\% reduction in assimilation and a significant increase in C isotope discrimination relative to controls (Ludwig et al., 1998). The reduction in assimilation was enhanced with increasing O$_2$ concentration, suggesting increased rates of photorespiration. All of these data are consistent with increased leakage of CO$_2$ from bundle sheath cells, and that this leakage is the result of over-expression of CA in this tissue.

**B. CAM Plants**

Our understanding of the role that CA plays in the functioning of CAM photosynthesis is very limited. With the exception of one study by Tsuzuki et al. (1982) on activities and localization of CA in a variety of CAM species, little has been done on this group of plants. This earlier paper showed that total CA activity for a variety of CAM species, such as \textit{Ananas comusus}, \textit{Sedum praealtum}, \textit{Hoya carnosa} and \textit{Mesembryanthemum crystallinum} were similar to many \text{C}_3 and \text{C}_4 plants but that localization varied. Plants which utilize PEP carboxykinase for decarboxylation contained only extrachloroplastic CA that partitioned with PEPCase during separation of intracellular compartments. In contrast, plants utilizing malic enzyme for decarboxylation contained chloroplast-localized CA activity. An additional observation was that facultative CAM plant \textit{Mesembryanthemum} exhibited no significant differences in either CA activity or localization when performing photosynthesis in either C$_3$ or CAM mode. Given what we now suspect about the role of CA in the mesophyll cell cytoplasm of \text{C}_4 plants, it is surprising that it is absent from this compartment in any one group of CAM plants. It may be that higher nocturnal CO$_2$ conductances in combination with lower levels of PEPCase in some CAM plants result in a requirement for less cytosolic CA than other CAM species. The apparent chloroplastic localization of CA activity in some CAM plants, such as \textit{Sedum}, also differs from the C$_4$ model. In these CAM plants, the chloroplastic CA and the resulting enhancement of C$_i$ equilibration may not impair decarboxylase-mediated CO$_2$ production as there are no additional cell layers for plasmodesmatal loss of HCO$_3^-$. In fact, loss of C$_4$ by CO$_2$ leakage may be reduced by rapid equilibration within the single cell of the CAM photosynthetic unit. The presence of chloroplastic CA may also be indicative of the amount of direct CO$_2$ diffusion, Rubisco-mediated fixation that takes place in CAM plants prior to malic acid synthesis and following decarboxylation. Direct CO$_2$ fixation by Rubisco can be an important component of the
total carbon assimilated during the diurnal cycle. It is interesting to note that a study by Maxwell et al. (1997) showed that the CAM plant Kalanchoe daigremontia exhibited extremely low levels of internal CO₂ conductance which reduced the efficiency of assimilation during phase IV photosynthesis in which atmospheric CO₂ is fixed directly by Rubisco. It was hypothesized that these low levels of CO₂ conductance were a consequence of the succulent nature of the thick leaves, with reduced air spaces and a densely packed mesophyll. The limited amount of CA that can be found in some CAM plant chloroplasts may be required to partially offset the restricted movement of CO₂ to the site of Rubisco carboxylation. It is apparent, however, that considerably more research is needed with CAM plants. Although an earlier study reported no changes in activity, it would seem that the well-characterized, NaCl induced C₃-CAM switching of Mesembryanthemum would be an ideal system for the molecular analysis of CA expression.

C. C₃ Plants

CA is an abundant polypeptide in the leaves of C₃ plants and may constitute upwards of 2% of the total soluble leaf protein. Although the bulk of the CA activity is associated with the chloroplast fraction, recent measurements have shown that at least 10 to 15% of the total activity is cytoplasmic (Rumeau et al., 1996). Even this higher than anticipated percentage of activity under-represents the significance of cytoplasmic CA unless one takes into account compartment volumes. As described in the paper by Rumeau et al. (1996) on subcellular distribution of CA in Solanum tuberosum leaves, assuming subcellular volumes of 9.5% and 3.4% for mesophyll cell stroma and cytosol, respectively. CA concentration in the chloroplast was only twofold higher than in the cytosol.

The CA isoform located in the chloroplast is synthesized as a larger precursor polypeptide in the cytosol and is then processed to the mature monomeric form. Recent studies have shown that this processing is a consequence of the sucrose nature of the thick leaves, with reduced air spaces and a densely packed mesophyll. The limited amount of CA that can be found in some CAM plant chloroplasts may be required to partially offset the restricted movement of CO₂ to the site of Rubisco carboxylation. It is apparent, however, that considerably more research is needed with CAM plants. Although an earlier study reported no changes in activity, it would seem that the well-characterized, NaCl induced C₃-CAM switching of Mesembryanthemum would be an ideal system for the molecular analysis of CA expression.

CA is an abundant polypeptide in the leaves of C₃ plants and may constitute upwards of 2% of the typical C terminal regions of other transit peptides. Careful in vitro chloroplast import studies with partial, full length, or chimeric Pisum sativum CA constructs have shown that it is only the N-terminal region that is required for correct transport, processing and localization in the chloroplast stroma (Forsman and Pilon, 1995). This study also provides some evidence for two step processing of the nascent CA during or following transport into the chloroplast. No specific role for the C-terminal region of the transit peptide has been shown.

First evidence for a cytosolic CA in C₃ plants was presented by Kachru and Anderson (1974) who reported on the partial purification of cytosolic and chloroplastic forms of CA in Pisum. Purification of the Solanum cytosolic isoform has shown that it is structurally and biochemically similar, and immunologically related to the chloroplastic form, although its monomeric mass is somewhat larger (30 kDa vs. 27 kDa), respectively (Rumeau et al., 1996). Cytosolic and chloroplastic isoforms are also present in Arabidopsis and cDNAs encoding each enzyme have been characterized (Fett and Coleman, 1994). Comparison of the deduced aa sequences revealed approximately 85% identity, however as expected, the cDNA encoding the cytosolic enzyme was missing a 78 aa N-terminus transit peptide sequence found on the chloroplastic isoform cDNA. Molecular masses of the two processed monomers are similar to that observed in Solanum, with the cytosolic isoform somewhat larger than the chloroplastic enzyme. Whereas two distinct nuclear genes encode the CA isoforms in Arabidopsis, in Pisum, data suggest that the two CA isoforms result from differential transcription of a single gene and/or subsequent processing of its mRNA (Majeau and Coleman, unpublished).

Immunogold localization studies with antisera directed against the chloroplast CA isoform show that CA is randomly distributed throughout the cytosol of both Solanum and Pisum (Rumeau et al., 1996; Anderson et al., 1996). In the chloroplast, these studies have shown that the bulk of the label to be found in the stroma, but a significant fraction was also found associated with granal and stromal thylakoids. Little if any label was found in association with the chloroplast envelope. These electron micrographs showing the presence of chloroplastic β-CA associated with thylakoid membranes has provided some support for a hypothesized thylakoidal CA (Stemler, 1997 and references within). It has
been suggested that CA (perhaps a distinct isoform) is associated with higher plant, algal and cyanobacterial thylakoids, specifically Photosystem II, and is required for efficient electron transport. Although a single protein component has never been isolated, extensively washed thylakoids and Photosystem II-enriched membrane fragments have been shown to catalyze the reversible hydration of CO\textsubscript{2}, suggesting that this CA activity is the product of an intrinsic membrane protein. In the absence of any identified, novel CA proteins, it is possible that the hydration activity of washed membrane fragments simply represents contamination by the abundant, soluble enzyme. Until a distinct CA isoform has been biochemically and genetically characterized, the presence and role of a thylakoidal CA remains an unresolved issue. Perhaps a cryptic isoform, either a novel α- or γ-type enzyme resides in the thylakoids and has yet to be characterized.

Another possibility for the cyto-immunological identification of CA in close proximity to thylakoids is that a portion of the CA may be a component of a complex with other Calvin cycle enzymes that form a loose association with photosynthetic lamellae. A number of studies have shown that enzymes such as Rubisco, phosphoribulokinase (PRK), glyceraldehyde phosphate dehydrogenase (GAPDH), ferredoxin-NAD\textsuperscript{+} reductase (FNR), and ribose-5-P-isomerase (RPI) as well as others appear to form heteromeric complexes capable of CO\textsubscript{2} fixation (Süss et al., 1993 and references within). It has been hypothesized that such thylakoid-associated enzyme complexes would facilitate channeling of intermediates, and an association with NADPH and ATP generating sites (the thylakoids) would greatly improve the efficiency of substrate utilization. Recently, biochemical evidence has been provided to show that CA is associated with a Calvin cycle heteromeric enzyme complex in the chloroplasts of tobacco (Jebanathirajah and Coleman, 1997). Following non-denaturing FPLC separation of stromal extracts, CA, Rubisco and other Calvin cycle enzymes were found to co-purify in the same fractions. Trypsin treatment followed by SDS-PAGE analysis of these purified complexes suggested that CA was localized on the periphery or in a less tight association than some of the intrinsic heteromeric complex proteins such as PRK and FNR (Jebanathirajah and Coleman, 1997). The presence of CA in an association with Rubisco certainly strengthens the argument that CA is involved in the direct provision of CO\textsubscript{2} to the C\textsubscript{3} Rubisco.

A description of location within the C\textsubscript{3} plant cell, numbers of isoforms, and possible association with other proteins leads logically to a discussion on the role of CA in C\textsubscript{4} higher plants. Although some progress has been made in this area, definitive evidence for any one role is still lacking. In all, three specific roles for the chloroplast-localized β-CA have been suggested. The first involves CA facilitating diffusion of CO\textsubscript{2} across the chloroplast envelope and maximizing the flux of C\textsubscript{i} through the alkaline stroma to the site of Rubisco-mediated carboxylation. As CO\textsubscript{2} appears to be the only C\textsubscript{i} species capable of crossing the chloroplast envelope, its diffusion from the more acidic cytoplasmic environment would be enhanced by the catalyzed hydration of CO\textsubscript{2} upon entry into the chloroplast. The rapid equilibration of the C\textsubscript{i} species to that point established by the pH of the stroma also ensures a maximal rate of C\textsubscript{i} flux through the stroma to the site of carboxylation. The rate of movement through the stroma is enhanced because both C\textsubscript{i} species are components of the gradient established by Rubisco-catalyzed CO\textsubscript{2} consumption (Cowan, 1986). The second major proposed role for CA is that which occurs at the site of carboxylation where catalyzed dehydration of the abundant HCO\textsubscript{3}\textsuperscript{-} pool in the alkaline stroma is presumed to maintain the supply of CO\textsubscript{2} for Rubisco. The association of a portion of the chloroplastic CA in a Rubisco-containing Calvin cycle complex is in support of this hypothesis. Certainly these two major roles for CA are not mutually exclusive and the spatial arrangement of CA in the chloroplast presumably complements both functions. The third hypothesized role for CA catalyzed CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} exchange in the stroma is that it is an important component in moderating rapid, localized pH changes induced by fluctuations in light intensity. The production or utilization of H\textsuperscript{+} during dehydration/hydration reactions could act as a biological buffering system, however, there is no experimental evidence to support or refute this hypothesis.

The confirmation of the existence of a relatively abundant cytoplasmic CA has also prompted some speculation as to a role for this enzyme. As has been suggested for the C\textsubscript{4} plant mesophyll CA, the primary function of a cytoplasmic C\textsubscript{4} CA may be to provide HCO\textsubscript{3}\textsuperscript{-} for PEPCase. PEPCase in C\textsubscript{4} plant mesophyll tissue plays an anaplerotic role in the biosynthesis of C\textsubscript{4} acids, and for maximal activity may require CA to speed hydration of CO\textsubscript{2}. In active guard cells, the
enhanced role of guard cell PEPCase would also require significant CA activity for HCO₃⁻ formation, however no guard cell-specific CA activity or localization measurements have been performed.

Recent attempts to identify specific roles for CA in C₃ plants have been based on methods to manipulate CA activity followed by an examination of plant growth or photosynthesis. Methods use to modulate CA activity have included growth of plants under Zn or N deficient conditions, use of sulfonamide inhibitors of CA with intact plants or isolated chloroplasts, and most recently, molecular technology employing transgenics expressing CA antisense constructs. Modulation of growth conditions by reducing the availability of N or Zn will reduce CA activity however the pleiotropic effects of these treatments and the high percentage of residual CA activity make the data from such studies difficult to interpret. Vacuum infiltration of the CA inhibitor ethoxyxylamide into C₃ plant leaf pieces has been used recently to improve the access of these inhibitors to the target tissue (Badger and Pfanz, 1995). In this study, CA inhibited Spinacea oleracea, Nicotiana rustica, and Hordeum vulgare leaf pieces exhibited 30 to 50% inhibition of photosynthesis at low external CO₂ concentrations. As levels of CO₂ increased, inhibition of photosynthesis was diminished. These data suggest that CA plays a relatively prominent role in photosynthetic carbon fixation. This is certainly different from observations with transgenic Nicotiana in which CA levels have been reduced by antisense expression. In two independent studies, growth and photosynthesis at air concentrations of CO₂ and high or low light levels were not markedly affected by levels of CA as low as 1% to 2% of wild type plants (Majeau et al. 1994; Price et al, 1994). Interestingly, ¹³C, discrimination values for on-line gas samples and for leaf dry matter of CA antisense plants were quite different from wild type plants. ¹³C tissue concentrations were consistently higher in low CA plants (Price et al., 1994; Williams et al., 1996). In combination with gas exchange analysis, these data indicated that chloroplastic CO₂ concentrations were approximately 15 to 20 μmol.mol⁻¹ lower in plants with low CA levels. The relatively small decrease in intracellular CO₂ would not result in a significant or easily observed decline in photosynthesis. Some initial data (Majeau et al., 1994) did suggest that low CA plants were compensating by increasing stomatal conductance to improve CO₂ entry into the leaf however this finding was not confirmed in a more detailed gas exchange study of these plants (Williams et al., 1996). To further confound this issue, however, there is at least one published report of Arabidopsis antisense CA plants being unable to grow in the absence of sucrose or high levels of external CO₂, suggesting that in some plants a reduction in CA activity can have a profound influence (Kim and Bartlett, 1996)

The isolation of null isoform-specific CA mutants would be an ideal strategy for further study of the role of CA in C₃ plants. Antisense or co-suppressed plants will always have some residual activity which may be sufficient for growth and photosynthesis under most conditions. In addition, the antisense constructs used to date are not isoform or tissue specific, again making the interpretation of any recognized phenotype somewhat difficult. One possibility would be to screen mutagenized populations of Arabidopsis to look for a null allele, however, electrophoretic or chromatographic separation and activity determination of the two isoforms would be necessary. Simple activity analysis of crude leaf preparations will only identify a mutant deficient in both isoforms, an extremely unlikely event.

Regulation of carbonic anhydrase expression in C₃ plants has been studied in a number of species, primarily in terms of activity modulation under various environmental conditions. Certainly the availability of Zn influences activity levels but it is only under severe Zn deprivation that CA levels are reduced and as such it is not likely to be a true regulating agent in vivo. Both N and CO₂ availability influence CA expression in concert with Rubisco expression. In most C₃ plants as leaf nitrogen values increase, both Rubisco and CA levels in plants (with the exception of Triticum) increase proportionally (Makino et al., 1992). This increase in carboxylation capacity, however, is not completely realized as enhanced in vivo rates of CO₂ fixation. As well as electron transport limitations on the production of RuBP, resistance to CO₂ flux from outside the leaf through to the substomatal cavity, and CO₂ transfer resistance between the intercellular airspaces and the carboxylation sites limits the availability of CO₂ (Evans and von Caemmerer, 1996). Even as CA levels increase (in concert with Rubisco), the partial pressure of CO₂ at the carboxylation sites is lowered by the increased abundance of Rubisco. These data suggest that although increased CA levels occur, they alone are unable to maintain CO₂ concentrations
within the chloroplast. It is possible that an increased CA/Rubisco ratio would be more effective in maintaining chloroplast CO₂ levels, however it is more likely that the intercellular air space transfer resistance and/or stomatal conductance play a more prominent role in limiting CO₂ flux, and thus in vivo Rubisco activity. Reduced levels of N or elevated (above ambient) levels of CO₂ result in declines in both Rubisco and CA activities, protein and transcript abundance. Conversely, the transfer of plants to elevated N levels or from elevated to ambient CO₂ concentrations enhances Rubisco and CA expression. The response to CO₂ can be quite rapid with an over three-fold increase in both chloroplast CA (ca) and Rubisco small sub-unit (rbcS) transcript abundance occurring in Pisum within three to six hours after transfer from 1000 to 350 μmol mol⁻¹ CO₂ (Majeau and Coleman, 1996). CA expression is even greater at below ambient levels of CO₂, conditions under which Rubisco levels decline, with the result that a considerable enhancement of the CA/Rubisco ratio occurs (Majeau and Coleman, 1996). Modulation of CA expression (and Rubisco) in response to elevated levels of CO₂ is presumably mediated by changes in carbohydrate levels and/or metabolism. Actual mechanisms or signaling agents have not yet been identified although considerable evidence supports the idea that specific hexokinase isoform activity is involved in carbohydrate induced down-regulation of transcription (Jang et al., 1997). The capacity for enhanced CA activity at less than ambient levels of CO₂, conditions which seem to repress rbcS transcript levels, suggests that CA expression may respond differentially to very low levels of carbohydrates or additional signals, perhaps CO₂ itself. In addition to N and CO₂ in Pisum, both chloroplastic CA and Rubisco expression at the levels of transcript and protein abundance are coordinated during leaf ontogeny and in response to light (Majeau and Coleman, 1994). This coordination of CA and Rubisco activity has also been described in cultivars of rice which exhibit different rates of leaf photosynthesis (Sasaki et al., 1996).

D. Cyanobacteria and Microalgae

Studies on regulation of CA expression and role in photosynthesis of aquatic microorganisms are numerous and have been extensively reviewed in previous years (Aizawa and Miyachi, 1986; Tsuzuki and Miyachi, 1989; Coleman, 1991; Sultemeyer et al., 1993; Badger and Price, 1994). In this section I will provide a general overview of the area and focus on recent research efforts and existing gaps in our knowledge.

The aquatic environment impacts significantly in a number of ways on the supply of CO₂ to the site of carboxylation. The rate of CO₂ diffusion in water is slower than in air by a factor of 10⁴; pH values above 7 result in HCO₃⁻ becoming the predominant C₄ species with the concentration of CO₂ in air equilibrated water remaining at 10 to 12 μM. In addition, the slow, uncatalyzed rate of dehydration of the external HCO₃⁻ pool (particularly at alkaline pHs) restricts its use in the provision of the species for CO₂-requiring reactions. As a result, many (if not all) aquatic photosynthetic microorganisms have developed CO₂ concentrating mechanisms (CCMs) (some induced by limiting C₄) which are capable of active transport of CO₂ and/or HCO₃⁻ and the formation of an intracellular C₄ pool. High concentrations of CO₂ derived from the intracellular C₄ pool provide sufficient substrate for Rubisco that the oxygenase reaction is minimized and little if any photorespiration occurs. The efficiency of the CCMs is revealed by the low affinity of the microalgal and cyanobacterial Rubisco which exhibit kₘ,CO₂ values ranging from 50 μM for Chlamydomonas to over 200 μM for Synechococcus. Apparently, the delivery of CO₂ to the site of carboxylation is sufficiently effective that there has been little need for selection of a high affinity Rubisco (as in terrestrial C₄ plants). Along with a reduced affinity for CO₂ of the algal and cyanobacterial Rubisco there has been a corresponding increase in Rubisco kₘ values with the result that, in concert with efficient CCM activity, many of these organisms can exhibit very high levels of productivity over a wide range of growth conditions.

What is the role of CA in the activity of cyanobacterial and microalgal CCMs and photosynthesis? It is well established that cyanobacteria and microalgae express higher levels of CA activity when transferred from replete to limiting concentrations of C₄, conditions which maximize the expression of CCMs. The cellular localization and role of CA within the CCMs and photosynthesis, however, can vary among groups of organisms. In the unicellular cyanobacterium Synechococcus PCC 7942, which has become the model organism for much research in this area, a β-type CA, the product of the icfA (ccaA) gene (Fukuzawa et al., 1992; Yu et
al., 1992) has been identified and appears to be associated with carboxysomes (Price et al., 1992). Carboxysomes are protein membrane encapsulated Rubisco aggregates found in cyanobacteria (and some other photosynthetic prokaryotes) and are the primary sites of CO\textsubscript{2} fixation. Both carboxysome number and CA levels increase following growth at limiting concentrations of CO\textsubscript{2} (Price et al., 1992; McKay et al., 1993). Mutants deficient in either carboxysome formation or icfA expression, although still able to transport and generate an intracellular pool of CO\textsubscript{2}, are unable to utilize this pool efficiently and require high external levels of CO\textsubscript{2} for growth (Fukuzawa et al., 1992; Yu et al., 1992; Price et al., 1993). The icfA gene product (and carboxysomes) are presumed to be an integral part of the cyanobacterial CCM. CA is thought to speed the dehydration of HCO\textsubscript{3}\textsuperscript{-} that diffuses into the carboxysome such that the CO\textsubscript{2} generated is in close proximity to the active Rubisco. The close association of icfA gene product and Rubisco minimizes the leakage of CO\textsubscript{2} out of the cyanobacterial cell however some loss does occur.

Additional CAs or CA-like proteins/activity are also found in cyanobacteria. One intriguing but still cryptic protein is also a suspected component of the cyanobacterial carboxysome. Although not known to have CA activity, the ccmM gene product of Synechococcus PCC 7942 is a 58 kDa polypeptide with considerable sequence similarity in the N-terminal region to the trimeric CA isolated from the archaebacterium Methanosarcina thermophila. The ccmM gene product also displays some limited sequence similarity to the small sub-unit of Rubisco including key residues required for binding of the small sub-unit to the large sub-unit of Rubisco (Price et al., 1993). Insertional inactivation of the ccmM gene results in cell lines which require high CO\textsubscript{2} concentrations for growth, although the potential disruption of carboxysome structure by such an inactivation strategy is also known to generate this phenotype (Price et al., 1993). Assuming a carboxysomal protein shell location for this protein, it is possible that it facilitates movement of HCO\textsubscript{3}\textsuperscript{-} into the carboxysome. The phenotype of cyanobacterial mutants generated by site-specific mutation constructs in which any catalytic capacity of the ccmM encoded protein is inactivated but coherent transcription/translation of it and the surrounding operon still occurs may yet answer questions on the role of this protein.

Extracellular (presumably periplasmic) \(\beta\)-type CAs have also been identified in cyanobacteria (Soltes-Rak et al., 1997; G. Espie, unpublished). Again, their role (if any) in the CCM remains cryptic. Although expression appears to be modulated by external CO\textsubscript{2} levels, insertional inactivation of the genes encoding these proteins results in only minor phenotypic differences when the cell lines are compared with wild type cells grown at low, ambient or elevated CO\textsubscript{2} concentrations (Soltes-Rak et al., 1997). It is possible that the primary role of these extracellular CAs may be as sensors rather than catalytic elements, and as such they would be the initial component of a signal transduction pathway that allow the cyanobacteria to rapidly respond to changes in extracellular CO\textsubscript{2}. There is some evidence to suggest that cyanobacteria and microalgae can directly sense and respond to changing CO\textsubscript{2} levels independently of photosynthetic carbon metabolism. Proteins, such as CA which directly interact with CO\textsubscript{2} or HCO\textsubscript{3}\textsuperscript{-} molecules, could play a role in such a signaling system. An unidentified CA-like moiety is also assumed to be an integral component of the cyanobacterial CCM. The activity of a ethoxazolamide-sensitive, presumably membrane-associated protein results in the conversion of CO\textsubscript{2} to HCO\textsubscript{3}\textsuperscript{-} prior to or during transport across the cytoplasmic membrane. The isolation and characterization of a putative vectorial CA, if it exists, would be a major step in our understanding of inorganic transport systems in aquatic organisms.

For the eukaryotic microalgae, it is primarily studies of CCMs of the green algae Chlamydomonas and Chlorella that have advanced our understanding of the roles that CA can play in this process. Indeed, the first well characterized CA from a photosynthetic organism and the first protein associated with the acclimation of algae to limiting CO\textsubscript{2} concentrations was the periplasmic localized \(\alpha\)-type CA of Chlamydomonas reinhardtii. Both transcript and protein levels encoded by cah1 increase several fold following transfer of cells from surfeit to limiting levels of CO\textsubscript{2}, and this pattern of expression accompanies the induction of the high affinity CO\textsubscript{2} transport systems (Bailly and Coleman, 1988; Fukuzawa et al., 1990). An additional extracellular CA, the product of cah2, is expressed at much lower levels and in a reciprocal fashion to cah1, with transcript and protein present only at high levels of external CO\textsubscript{2} (Fujiwara et al., 1990; Rawat and Moroney, 1991). Although not absolutely required for either CO\textsubscript{2} or HCO\textsubscript{3}\textsuperscript{-} transport, the presumed role
of the extracellular CAs is to maintain the external
CO$_2$/HCO$_3^-$ equilibrium and thus ensure the avail-
ablety of either C$_i$ species for the respective
transporters.

The study of intracellular CAs in microalgae has
been a complicated issue. There were many earlier
reports of various soluble and insoluble CA isoforms
exhibiting differing responses to CA inhibitors and
CO$_2$ levels, and localized in both the cytoplasm and
chloroplast (Sultemeyer et al., 1993 and references
within; Husic and Marcus, 1994; Amoroso et al.,
1996), however, definitive protein or molecular
evidence for many of these isoforms was lacking.
Certainly some variation in data from the various
research groups could be the result of numbers of
different organisms and isolation procedures used,
but it is only recently that substantive and compelling
evidence for two specific intracellular isoforms has
been presented. A novel $\alpha$-type CA ($cah3$ gene
product) has been isolated and characterized, and in
the initial study the CA appeared to be localized in the
Chlamydomonas chloroplast (Karlsson et al.,
1995). These data suggested perhaps an association
with Rubisco in the pyrenoid (a starch-sheathed
proteinaceous complex), where the CA would
catalyze the supply of CO$_2$ from the intracellular
HCO$_3^-$ pool for fixation. The postulated association
of Rubisco and CA in the pyrenoid would be
analogous to that which is thought to occur in the
cyanobacterial carboxysome, where CA and Rubisco
are also co-localized. In a later study, it was shown that
Chlamydomonas mutants exhibiting a high CO$_2$
requiring phenotype were genetically complemented with the
$cah3$ gene, thus helping to define a role for
this CA in Chlamydomonas (Funke et al., 1997).
In a recent study, it has now been shown that the $cah3$
gene product is in fact found in the chloroplast, but
localized to the thylakoid lumen of the Chlamy-
domonas chloroplast, and is required for growth at
ambient levels of CO$_2$ (Karlsson et al., 1998).
Expression appears to be regulated by C$_i$ levels, with
CA activity increasing at low levels of extracellular
C$_i$. It has been hypothesized that within the lumen,
CA would speed the dehydration of HCO$_3^-$
transported into the lumen during light-induced proton
uptake, and thus generate CO$_2$ for Rubisco catalyzed
fixation. CA-containing thylakoids which traverse
the Rubisco-rich pyrenoid may provide the colocalization
needed for the efficient coupling of the
intracellular HCO$_3^-$ pool and the requisite formation
of CO$_2$.

In addition to the $\alpha$-type chloroplast CA, a new $\beta$
type CA localized in the mitochondria of Chlamy-
domonas has been characterized (Eriksson et al.,
1996). Two highly homologous nuclear genes ($ca1$
and $ca2$) encode two identical 20.7 kDa proteins
which are both expressed following transfer of the
cells to limiting CO$_2$ concentrations. The postulated
role for this enzyme is to assist in stabilizing
mitochondrial pH. Following transfer of the cells to
low CO$_2$ concentrations and prior to the full induction
of the algal CCM, there is an increased flux of
photorespiratory intermediates produced by Rubisco
operating in a CO$_2$ limited environment. Mitochon-
drial decarboxylation of glycine may produce
sufficient amounts of NH$_3$ that alkalization of the
mitochondrial matrix could occur. CA activity would
ameliorate this problem by speeding the hydration of
photorespiratory CO$_2$ and the concomitant production
of H$^+$ (Eriksson et al., 1996). It is interesting to note
that another study has described changes in
mitochondria location in Chlamydomonas cells
acclimating to limiting CO$_2$ concentrations and have
identified by immunogold labeling a mitochondria-
localized polypeptide synthesized in response to low
CO$_2$ (Geraghty and Spalding, 1996).

An intriguing and unknown element in the C$_i$
regulation of CA (and the CCMs) expression in
microalgae and cyanobacteria is the nature of the
inducing signal. It had long been supposed that
various carbon metabolite levels, presumably
photorespiratory in origin, increased when cells were
transferred from surfeit to limiting C$_i$ concentrations
and that this organic carbon flux triggered the
induction of CA along with CCM expression
(Coleman, 1991 and references within). The reported
absolute requirement for light and photosynthesis,
as well as the O$_2$ sensitivity of the induction process
all supported this argument. There were also, however,
reports showing that induction of Chlamydomonas
$cah1$ transcript and protein could also occur when
cells were transferred to limiting CO$_2$ concentrations
in the dark, and in Chlorella, CA activity could be
induced at low CO$_2$ in the presence of the electron
transport inhibitor DCMU or very low light (Ballay
and Coleman, 1988; Shiraiwa and Miyachi, 1983).
Recently, these earlier reports have been comple-
mented by additional studies that show that $cah1$
expression in Chlamydomonas can be circadianly
entrained (Fujiwara et al., 1996), and can be induced in
the dark following transfer from high to limiting
CO$_2$ concentrations in both synchronous (Rawat and
Moroney, 1995) and asynchronous cultures (Villarejo et al., 1996). Studies with Chlorella ellipsoidea have also shown that induction of the CCM occurs in response to external C\textsubscript{i} concentrations, not intracellular C\textsubscript{i} levels, and that light speeds but is not required for CCM induction (Matsuda and Colman, 1995). Data obtained from a novel set of experiments in which the ca1/ca2 promoter region of the mitochondria-localized CA was ligated to the arylsulfatase reporter gene and used to transform Chlamydomonas support these observations (Villand et al., 1997). As expected, arylsulfatase expression was induced by limiting CO\textsubscript{2} concentrations (as is wild type ca1/ca2 expression) but was not repressed by exposure to low O\textsubscript{3} concentrations and limiting C\textsubscript{i} levels. These conditions should limit the production of photorespiratory metabolites, and yet CA expression still occurred. Such an experimental system is ideal for more detailed promoter analysis and characterization of both cis and trans elements required for C\textsubscript{i} sensing and CA expression. In addition, using this reporter system in combination with Chlamydomonas mutagenesis it should be possible to determine the entire signal transduction pathway for acclimation to external CO\textsubscript{2} in microalgae. The elucidation of algal mechanisms of CO\textsubscript{2} sensing and response may also lead to a better understanding of CO\textsubscript{2} responsive elements in both higher plants and cyanobacteria.

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Chapter 16

CO₂ Acquisition, Concentration and Fixation in Cyanobacteria and Algae

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Summary

Aquatic photosynthetic organisms face a number of unique problems with regard to the supply of CO₂ for photosynthesis. These stem largely from the physical chemistry of the water phase in which they live, where the diffusion of CO₂ species is slow and CO₂ can exist as both CO₂ and HCO₃⁻ depending on the pH of the medium. Given the constraints, a number of solutions have evolved to optimize photosynthetic CO₂ fixation in algae and cyanobacteria. The two chief strategies that are apparent are the development of CO₂-concentrating mechanisms based on the active uptake of both CO₂ and HCO₃⁻ and the evolution of more efficient forms of Rubisco which are able to fix CO₂ at limiting levels of CO₂. This chapter examines aspects of co-evolution of Rubisco and CO₂-concentrating mechanisms in both algae and cyanobacteria. Particular emphasis is placed on what is known about the mechanism of the operation of carbon concentrating mechanisms (CCMs) in cyanobacteria and green microalgae. In cyanobacteria, multiple active C₅ transporters drive the CCM on the plasma membrane. These are energized by photosynthetic ATP and NADPH production, with the NAD(P)H dehydrogenase complexes playing a critical role. A pool of HCO₃⁻ is accumulated within the cell and this is used by the Rubisco-containing carboxysome to generate CO₂ within this localized micro-environment. Carboxysomal carbonic anhydrase is crucial to this CO₂ generation process. For green microalgae, active C₅ transport occurs at both the plasma membrane and the chloroplast envelope. A HCO₃⁻ pool is accumulated in the chloroplast stroma, with the aid of photosynthetic energy, and this pool is used to elevate CO₂ around Rubisco. Rubisco is primarily localized to the pyrenoid. A crucial part of this conversion appears to be a thylakoid lumen carbonic anhydrase, which may use the luminal protons to drive this process. The CCM is inducible in nature in both cyanobacteria and algae, increasing its affinity for external C₅ when cells are grown at limiting C₅ conditions. In both green and non-green algae, the presence of significant CCM activity is correlated with the presence of pyrenoids and single chloroplasts within cells. This chapter examines the possible diversity of CCM operation among the green and non-green algae, highlighting possible variation in CCM operations compared to the models developed for green microalgae. Considerable work remains to be done to identify specific variation of CCM mechanisms in non-green algae.

I. Supply of CO₂ in an Aquatic Environment

Algae and cyanobacteria undertake photosynthesis in an aquatic environment where they face a number of unique problems regarding the efficient operation of photosynthesis. Among these, the acquisition of CO₂ from the external medium and its supply as substrate for the primary CO₂-fixing enzyme Rubisco (EC 4.1.1.39) has been of great significance. The evolution of terrestrial plants was a major step in altering the limitations imposed by these aquatic problems. Air filled leaves reduced the effective aqueous diffusion path to a few micrometers and the photosynthetic cells have rapid access to the CO₂ existing in the bulk atmosphere (Raven, 1970; Kerby and Raven, 1985; Raven et al., 1985). Since the diffusion coefficient for CO₂ in air is some 10⁴ higher than in water, this greatly reduces the problems of CO₂ supply to Rubisco.

The nature of CO₂ supply problems and opportunities facing an aquatic photosynthetic cell stem largely from the physical chemistry relating to inorganic carbon (C₅) species in solution. Figure 1 depicts aspects of this supply problem. There is relatively slow equilibration of CO₂ between the air and the water, which can result in a depletion of the water [C₅] during conditions of active photosynthesis (Talling, 1985; Adams, 1985). In solution, all species of C₅ including CO₂, HCO₃⁻ and CO₃²⁻ diffuse relatively slowly, so if unstirred layer thicknesses are significant then draw-down can occur between the bulk water and the cell surface. For small cells such as microalgae and cyanobacteria, the diffusion path through the unstirred layer (l) is very small (less than a few μm) so unstirred layer thicknesses are not a limitation. However, this is not so for macrophyte algae which have much larger multicellular dimensions (Kerby and Raven, 1985).

Perhaps the most significant feature of the aquatic

Abbreviations: CA – carbonic anhydrase; CCM – CO₂-concentrating mechanism; C₅ – inorganic carbon (CO₂ + HCO₃⁻); DCMU – dichlorodiphenylmethy urea; PEP – phosphoenolpyruvate; PGA – 3-phosphoglyceric acid; RuBP – ribulose 1,5-bisphosphate; Rubisco – ribulose bisphosphate carboxylase/oxygenase; Sₑₑ – relative specificity factor for Rubisco relating the carboxylase to oxygenase reaction kinetics (Vₑₑ*Kₑₑ)/(Kₑₑ*Vₒₒ).
system are that all species of \( C_4 \) (\( CO_2 \), \( HCO_3^- \), and \( CO_3^{2-} \)) are interconverted by a series of hydration, dehydration and protonation reactions as described in Fig. 2. The significant features of this chemistry are that firstly the ratio of \( CO_2 \) to \( HCO_3^- \) in solution is highly dependent on the pH of the aquatic environment and secondly, the hydration of \( CO_2 \) to \( HCO_3^- \) is very slow compared to the use of \( CO_2 \) by photosynthesis (Badger and Price, 1994). The consequences of this chemistry are two-fold. Firstly, \( HCO_3^- \) becomes the dominant \( C_4 \) species in solution above pH 6.3. This is particularly so in marine environments where the pH is above 8, \( HCO_3^-/CO_2 \) is \( > 50 \) and the \([HCO_3^-]\) is around 1 mM compared to 10 \( \mu \text{M} \) for \([CO_2]\). Secondly, in environments where photosynthesis is relatively rapid per unit volume (either in the bulk phase or unstirred layers) both the slow conversion of \( HCO_3^- \) to \( CO_2 \) and solubilization of \( CO_2 \) from the air can cause a significant depletion of \( CO_2 \) below its air equilibrium value. Accompanying rises in pH in relatively unbuffered water bodies (Adams, 1985; Spence and Maberly, 1985; Talling, 1985) exacerbates this.

As will be described later, both algae and cyanobacteria have developed mechanisms to enable both \( CO_2 \) and \( HCO_3^- \) to be used to support photosynthesis. For some cells, this has been achieved by direct transport of \( HCO_3^- \) across otherwise impermeable lipid cell membranes while others are able to use variable amounts of \( CO_2 \) either by passive diffusion into the cell or active uptake. For the uptake of \( CO_2 \), the frequent prevalence of extracellular CA in algae, in particular, allows these cells to make rapid use of the external \( HCO_3^- \) pool and overcome its chemically slow dehydration to \( CO_2 \) (see Badger and Price, 1994).

II. Efficient \( CO_2 \) Capture Mechanisms Evolved Following Changes in Atmospheric \( CO_2 \) and \( O_2 \)

The process of photosynthetic \( CO_2 \) fixation has evolved over time in response to a number of environmental constraints and photosynthetic organisms have presumably developed to optimize their efficiency under a range of environmental conditions. In particular, the extent to which \( CO_2 \), the primary substrate for Rubisco, operates as a limiting factor has changed dramatically over the time span of evolution. In view of this, it is not surprising that
there has been diverse adaptation by organisms in the mechanisms that have developed to alleviate the limitation which CO₂ availability imposes on photosynthesis.

The primary CO₂ fixing enzyme in all photosynthetic organisms, Rubisco, appears to have arisen once in the evolution of life, appearing with the chemolithotrophs, which arose some 3.5 x 10⁹ years ago (Broda, 1975). In these environments, the CO₂ level was high and the O₂ was very low. Carbon dioxide was unlikely to have presented a substrate limitation to Rubisco and it is likely to have operated close to substrate saturation in these organisms. In addition, the deleterious effects of the oxygenase activity of Rubisco would not have been significant and photorespiration was unlikely to have been a problem.

Eventually, however, the operation of CO₂ consuming processes, particularly Rubisco carboxylation, led to a decline in the level of atmospheric CO₂, particularly in the carboniferous era some 350 million years ago (Berner, 1990,1993). Furthermore, with the advent of cyanobacteria and oxygenic photosynthesis, the O₂ levels began to rise. The result of these changes was that the CO₂/O₂ ratio fell dramatically and the atmospheric and aquatic environments became progressively less favorable for carboxylation by Rubisco. Primitive Rubiscos had poor kinetic properties with respect to their affinity for CO₂ and relatively high oxygenase potentials (Badger and Andrews, 1987; Badger et al., 1998). Eventually, net CO₂ fixation would have been threatened if changes had not occurred to either the properties of the Rubisco or the manner in which CO₂ was supplied to Rubisco from the external environment.

Not unexpectedly then, photosynthetic organisms have adapted to the changing CO₂ conditions of the environment in two ways. Firstly, Rubisco itself has shown considerable ability to evolve by increasing its affinity for CO₂ (Kₘ,CO₂) and decreasing the relative activity of the oxygenase reaction, as evidenced by a higher Sₘₐₓ value (see section below). Secondly, organisms have developed mechanisms to insulate Rubisco from the external environment where the CO₂ levels are actively elevated (Badger, 1987).

If a broad range of aerobic photosynthetic organisms is considered, including cyanobacteria, algae and higher plants, then it appears that the majority have developed some form of CO₂-concentrating mechanism (CCM) which aids the performance of Rubisco. In higher plants, the most obvious example is the development of C₄ photosynthesis, but functionally equivalent mechanisms have also developed in algae and cyanobacteria. Although there are significant differences between the particular details of each CCM, comparisons show that there are similar functional elements of CCMs across all systems. Table 1 and Fig. 3 present a comparison of these functional CCM components in C₄ higher plants, algae and cyanobacteria. The common components between cyanobacteria and algae include: active Ci transport systems, energized by photosynthesis; an internal pool of Ci which is the source of CO₂ for Rubisco; a compartmentalized Rubisco, in either carboxysome or pyrenoid, together with carbonic anhydrase that generates CO₂ from HCO₃⁻; a barrier to the efflux of CO₂.

In all CCMs, the balance between the rate at which CO₂ is produced in the localized Rubisco compartment and the rate at which it leaks back to the external environment or is fixed by Rubisco determines the extent to which CO₂ is elevated around Rubisco. Detailed aspects of the operation of the CCMs in cyanobacteria and algal species will be explored more fully later in this chapter.

III. Co-evolution of CCMs and Rubisco

In recent years, evolutionary and biochemical studies of Rubisco have given some new and exciting insights into the events which have led to the distribution of different Rubisco lineages among the algae, cyanobacteria and higher plants. The current evolutionary tree of algal and cyanobacterial Rubiscos is summarized in Fig. 4. It is evident that Rubisco shows an evolutionary lineage that is quite different to the light harvesting machinery of the thylakoids (Delwiche and Palmer, 1996; Watson and Tabita, 1997).

Within algae, cyanobacteria and higher plants, there are at least 3 distinct types of Rubisco enzymes. Form 1 LₐSₜ Rubisco forms dominate algal and higher plant chloroplasts, but there are distinct differences among these enzymes. Most of the cyanobacteria, chlorophyte algae and higher plants possess what has been termed the green Form 1 type enzymes with evolutionary lineage from the cyanobacterial Form 1 enzyme. Interestingly, it has been recently recognized that some cyanobacteria (e.g. Synechococcus WH7803) possess a green Form
1b enzyme with evolutionary linkages to the enzyme from \( \beta / \gamma \) proteobacteria such as *Chromatium vinosum* (Watson and Tabita, 1996). The non-green algae, containing Chrysophytes, Rhodophytes and Phaeophytes, are distinctly different from the Chlorophyte algae. They possess a Red Form I Type Rubisco, with evolutionary linkages to the \( \alpha / \beta \) purple proteobacteria such as *Alcaligenes eutrophus*. Dinoflagellates such as *Amphidinium carterae* are much more divergent than other non-green algae, possessing an \( L_{\delta} \) Form II Rubisco enzyme, related to proteobacteria such as *Rhodospirillum rubrum*.

The existence of such distinctly different forms of Rubisco enzymes within the algae has implications when considering the evolution of strategies to acquire CO\(_2\) from the surrounding medium. Figure 4 also summarizes the measured kinetic properties for Rubisco enzymes across the algal species spectrum.
as well as for their ancestral relatives (for a more detailed review see Badger et al., 1998). Some salient points can be summarized. Firstly, despite the ancestral lineage of the Rubisco, there has been evolution of its kinetic properties to meet some of the constraints of its photosynthetic environment. Some examples of this can be seen in the following. For Dinoflagellates, the kinetic properties of the Form II enzyme (as measured by $S_{rel}$) are considerably improved over its proteobacterial ancestors. Such evolution is obviously necessary to allow the enzyme to function in an atmosphere of 21% $O_2$ and reduced $CO_2$. However, a CCM is an important part of Dinoflagellate photosynthesis suggesting that this Rubisco cannot operate effectively at air levels of $CO_2$ and $O_2$. The Form I enzyme from red algae such as *Cyanidium* and *Galdieria* (Uemura et al., 1997) have much higher $S_{rel}$ values and lower $K_m(CO_2)$ when compared to other red algal counterparts. This appears to be an adaptation to growth in high temperature acid hot-springs environments where there is definite pressure to evolve a Rubisco with a higher affinity for $CO_2$. In the green alga *Coccomyxa* (Palmqvist et al., 1995), a lower $K_m(CO_2)$ and higher $S_{rel}$ are seen compared to other Chlorophytes with CCMs, such as *Chlamydomonas*. This is clearly a response to adapt to an environment with a passive supply of $CO_2$ in these algae that lack a CCM.

The different Rubisco forms in the algae also have intrinsic properties that lead to different kinetic constraints, which may have to be dealt with by each group. Studies of both Rubisco kinetic properties and photosynthetic oxygen exchange (Badger et al., 1998) have shown that there are considerable differences in the potential oxygenase reactions exhibited by the Red and Green Form I types. In general, Red Types have less potential for oxygenase activity. This is largely due to a much-reduced affinity of the oxygenase reaction for $O_2$ and may in some cases be due to a change in the ratio between carboxylase and oxygenase $V_{max}$. This is summarized in Fig. 5 for four different Form I enzymes from algal, cyanobacterial and higher plant sources. Thus, following the evolution of the Chlorophyte Rubisco

![Fig. 4. A phylogenetic tree showing the evolutionary relationships between Rubisco enzymes found in photosynthetic organisms, together with their kinetic properties. The phylogenetic tree is adapted from Delwiche and Palmer (1996), and the kinetic parameters and CCM characteristics are taken from data summarized by Badger et al. (1998).](image-url)
into higher plants, although it has increased the affinity for CO$_2$, it has also increased the affinity of the oxygenase reaction. This has led to a situation in which the higher plant chloroplast has to deal with potentially high rates of oxygenase reaction. This has been accommodated by the development of the photorespiratory cycle to recycle glycolate (Ogren, 1984). For non-green algae, the reduced oxygenase reaction (see Fig. 5) may mean that strategies to deal with glycolate synthesis are much less important. Interestingly, although the $S_{rel}$ value of cyanobacterial enzyme is low, it shows a poor affinity for oxygen, which means that at atmospheric levels of oxygen it displays low oxygenase activity. However, the $K_m$(CO$_2$) is also quite high in the Type 1a cyanobacterial Rubisco enzyme, with the consequence that cyanobacteria cannot function effectively without the aid of a CCM.

An interesting correlation with changes in $K_m$(CO$_2$) in Rubisco enzymes is that the $V_{max}$ of the enzyme per active site increases with higher $K_m$(CO$_2$) (Fig. 5). Thus, algae and cyanobacteria employing a CCM are able to achieve a relatively high rate of photosynthesis per unit Rubisco. A cyanobacterium with a CCM may achieve Rubisco carboxylase turnover rates of 11 s$^{-1}$·site$^{-1}$, while spinach in air would be less than 1. This may contribute to improved nitrogen use efficiency for these organisms which often grow in nutrient limited waters. Interestingly, the Red Type enzyme from *Phaeodactylum*, because of its high $S_{rel}$ and higher $V_{max}$, would actually appear to be better than the spinach enzyme at current air levels of
substrate CO₂ and O₂.

There has been considerable interest recently in the fact that non-green algae (such as reds and diatoms) may possess a Rubisco kinetically superior to the higher plant green Form I enzyme (Read and Tabita, 1994; Uemura et al., 1997). Interestingly, all non-green algal Rubiscos with high Sₚ values probably come from algae with some level of CCM. This includes Phaeodactylum, Porphyridium, and Cylindrotheca (see Badger et al., 1998). It appears that for these enzymes, the oxygenase reaction is not very relevant for the functional performance of the chloroplast, but the lower affinity (Kₘ(CO₂) 20–40 μM) for external CO₂ probably is. Thus a CCM must still be used to allow Rubisco to fix CO₂ efficiently. Further studies are needed to clarify the role of a CCM in non-green algae.

IV. Operation of CCMs in Cyanobacteria

A number of reviews dealing with cyanobacterial CCMs have appeared in recent years (Badger, 1987; Miller et al., 1990; Coleman, 1991; Kaplan et al., 1991; Price and Badger, 1991; Badger and Price, 1992; Kaplan et al., 1994), and readers are directed here for a detailed analysis of current research perspectives. The purpose of the description that follows is to summarize the current state of our knowledge and to point out the areas that are unresolved.

A generalized description of the operation and components of the CCM in cyanobacteria is shown in Fig. 6. Inorganic carbon is transported by a number of Cₙ transporters on the cell membrane and enters the cell as HCO₃⁻. This transport is energized by photosynthetic electron transport and probably involves both cyclic and linear electron transport. The concentrated cytosolic HCO₃⁻ pool enters the carboxysome where it is converted to CO₂ through the action of carboxysomal CA. CO₂ is elevated in the carboxysome environment through some property of the carboxysome that restricts CO₂ efflux. The analogy of this CCM to other CCM-systems is shown in Fig. 3 and Table 1.

A. Inorganic Carbon Transport

There are multiple Cₙ transport systems that appear to be associated with the plasma membrane of cyanobacteria. These transport systems have the ability to actively accumulate both CO₂ and HCO₃⁻ from the external medium (see reviews Miller et al., 1990; Badger and Price, 1992; Kaplan et al., 1994). However, despite what appears to be multiple transport systems, there is strong evidence to suggest that all transport processes deliver HCO₃⁻ to the cytosol of the cell. This leads to an elevation of the internal HCO₃⁻ pool up to 1,000-fold over that in the external environment (Badger and Price, 1992). Although both CO₂ and HCO₃⁻ can be actively transported, most cyanobacteria grow in environments where the pH is in excess of 7.5, and under these conditions (see Fig. 2), it is likely that HCO₃⁻ uptake is the dominant transport process contributing to Cₙ uptake.

Until recently, physical evidence for the existence of multiple transporters or any understanding of how they functioned has not existed and speculation has relied on a number of physiological experimental approaches. Multiple transporters were predicted several years ago, largely based on experiments examining the requirement for Na⁺ for Cₙ transport and the effects of various CO₂ and Na⁺ analogues on...
C₃ transport (see Miller et al., 1990). Evidence has been presented for what appears to be both Na⁺-dependent and Na⁺-independent HCO₃⁻ transport systems. The role of Na⁺ in C₃ uptake is unknown, but it is likely to be involved in pH regulation via Na⁺/H⁺ antiport mechanisms. However, a direct involvement of Na⁺ in a Na⁺/HCO₃⁻ symport system (Espie and Kandasamy, 1994) cannot be completely discounted. A separate CO₂ transport system is also apparent and this is inhibited by compounds such as COS and NaS (Miller et al., 1991). Interestingly, physiological analyses have also shown that there are a number of commonalities between the CO₂ and HCO₃⁻-transport systems. This includes inhibition by the carbonic anhydrase inhibitor ethoxyzolamide (Price and Badger, 1989a; Badger et al., 1994; Tyrrell et al., 1996) and a common requirement for a competent NADH dehydrogenase complex for what appears to be energization (Ogawa, 1990, 1991b, 1992). A final understanding of just how many transport components exist, how they operate and in what ways they are connected remains to be resolved. However, this understanding will almost certainly arise from molecular genetic approaches that are currently yielding new and exciting information about genes and proteins involved in C₃ transport systems.

Genetic evidence for multiple C₃ transporters has recently emerged, with the finding that the cmpABCD operon from Synechococcus PCC7942 codes for what appears to be a HCO₃⁻ transporter (Fig. 7). This transporter has characteristics which suggest that it is involved in high-affinity HCO₃⁻ uptake, it is induced under severe C₃ limitation and appears to be Na⁺-independent (Okamura et al., 1997; T. Omata and G. D. Price, personal communication). This work clearly indicates that there is at least one specific HCO₃⁻ transporter, and this appears to be highly analogous to a diverse sub-family of ABC (ATP Binding Cassette) transporters found in prokaryotes (see Price et al., 1998). The cmpA gene codes for the precursor of the 42 kD plasma membrane protein which was previously found to be induced under C₃ limitation (Omata and Ogawa, 1985, 1986) and high-light stress (Reddy et al., 1989). Analysis of cmpABCD mutants also confirms that this transport complex is only one of at least two HCO₃⁻ uptake systems (T. Omata and G. D. Price, personal communication). There is a significant Na⁺-dependent transport system, which appears more important at high-C₃ growth conditions, and there is a distinct CO₂ transport system that remains unaffected in the cmpABCD mutants. The existence of other HCO₃⁻ transport systems still remains a distinct possibility.

A potential structure for the cmpABCD HCO₃⁻ transporter is shown in Fig. 7. This is drawn by analogy with other ABC transporters which have been characterized (Higgins, 1992; Omata et al., 1993). CmpA codes for a periplasmically exposed HCO₃⁻ binding protein (42 kDa); cmpB is an intrinsic membrane protein and most probably forms a dimer within the membrane; cmpC is a large extrinsic membrane protein with an ATP binding site; cmpD is a smaller related protein also with an ATP binding site. The closest homologue of cmpC is part of the

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**ABC Bicarbonate Transporter**

![Diagram](image-url)

*Fig. 7. The operon and protein structure of the ABC-bicarbonate transporter characterized from Synechococcus PCC6803. The figure is modified from Omata et al. (1997).*
nitrate transporter, nrtC, which has been shown to be involved in regulation of activity in the presence of ammonia (Kobayashi et al., 1997). cmpC may also be involved in regulation of this ABC-type HCO$_3^-$ transporter. CmpA exists in stoichiometric excess to other components of the transporter (Omata and Ogawa, 1986), which is often a feature of binding proteins in ABC transporters (Higgins, 1992).

**B. Energization of $C_i$ Transport**

It appears that the operation of $C_i$ transport systems, both CO$_2$ and HCO$_3^-$ are highly dependent on the presence of light and photosynthetic electron transport. Experiments clearly show that there is little $C_i$ transport activity in the dark or in the presence of DCMU, and hence the involvement of respiratory energy sources is relatively insignificant in being able to energize the transport (Badger, 1987; Miller et al., 1990; Kaplan et al., 1991). Action spectrum studies have suggested that PS I driven cyclic electron transport was primarily responsible for the supply of energy, but some PS II activity is apparently required for activation of $C_i$ uptake (Ogawa et al., 1985; Kaplan et al., 1987). The existence of an ATP-using ABC-HCO$_3^-$ transporter would suggest that this transport process could be dependent on PS I driven cyclic ATP production, with activation in the light being regulated in some specific manner, perhaps being dependent on photosynthetic reductant. However, there is also evidence to suggest that whole chain electron transport can also support $C_i$ transport (Miller et al., 1991; Li and Canvin, 1997).

Analysis of mutants has also added to the picture of light-driven energization. The NAD(P)H dehydrogenase complex has been found to be essential for the functioning of both CO$_2$ and HCO$_3^-$ transport processes. Genetic elimination of particular subunits of this complex (ndhB K and L) (Ogawa, 1991a, 1992; Marco et al., 1993) had dramatic effects on eliminating both CO$_2$ and HCO$_3^-$ transport. These studies also led to the discovery that the NAD(P)H complex was intimately involved in mediating the reduction of the thylakoid plastoquinone pool using NAD(P)H reductant (Mi et al., 1992, 1994, 1995), thus catalyzing one form of cyclic electron transport around PS II. The obvious interpretation arising from these studies was that a thylakoid located NAD(P)H complex was involved in mediating the cyclic photophosphorylation which produced ATP to drive $C_i$ uptake by ATP-dependent $C_i$ transporters such as the then-unknown ATP-dependent ABC HCO$_3^-$ transporter.

Further studies of the NAD(P)H dehydrogenase mutants and gene expression have added complexity to the role of this complex in $C_i$ transport. Inactivation of a number of the subunits of this complex (ndhF and D in particular) apparently having more subtle effects on $C_i$ transport (Ohkawa et al., 1998; Price et al., 1998) appearing to have specific effects on reducing high-affinity CO$_2$ transport (Sültemeyer et al., 1997a; Ohkawa et al., 1998; Price et al., 1998). With the publishing of the complete genome for Synechocystis PCC6803 (Kaneko and Tabata, 1997; Nakamura et al., 1998), it has become apparent that both ndhD (6 homologues) and F (3 homologues) are members of a multigene family, while the other ndh genes are single copy. Furthermore, it is apparent that only one member of the ndhD and ndhF gene families may be involved in mediating the functioning of high-affinity CO$_2$ transport. This has been clearly shown for Synechococcus PCC7002 (Price et al., 1998) and Synechocystis PCC6803 (Ohkawa et al., 1998) and in Synechocystis, the ndhD3 gene shows specific upregulation of expression during growth at low CO$_2$ (Ohkawa et al., 1998).

The evidence is beginning to suggest that there may in fact be multiple NAD(P)H dehydrogenase complexes in the cell, with perhaps both thylakoid and plasma membrane locations (see Price et al., 1998). The complexes may have common core components, such as the ndhB, K and L, but there may be variability of some of the polypeptide components, such as the ndhD and F. This throws into question just which NAD(P)H complex is involved in energizing $C_i$ transport. The original ndhB, K and L mutants would have eliminated both plasma membrane and thylakoid complexes. So perhaps there is a role for plasma membrane NAD(P)H complexes which remains to be more fully described (Price et al., 1998).

Another gene product that appears to play a role in energizing $C_i$ uptake, at least in Synechococcus PCC7002, is the PsaE protein associated with PS I (Sültemeyer et al., 1997b). This protein appears to be involved in mediating an alternative type of PS I cyclic electron flow (Yu et al., 1993), and inactivation of psaE leads to a loss of high-affinity HCO$_3^-$ transport, which is developed at growth conditions.
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C. Cyanobacterial Carbonic Anhydrases

The model shown in Fig. 6 implies specific localization of carbonic anhydrase in the cyanobacterial cell. For this model to function there is a carbonic anhydrase associated with the carboxysome, and a specific absence from the cytosolic compartment. The *Synechocystis* PCC6803 genome sequence indicates that there are two potential carbonic anhydrase genes. One of them has close homology with what has been shown to be a carboxysomal CA (icfA)(Fukuzawa et al., 1992; Price et al., 1992b; Yu et al., 1992). The other has closest homology to the *Chlamydomonas* mitochondrial βCA, but its function seems cryptic. Inactivation of this gene in *Synechocystis* produces no discernible phenotype relating to the operation of the CCM (T. Ogawa and G. S. Espie personal communication). Adding to this evidence is the observation that expression of a human CA protein in the cytosol of *Synechococcus* PCC7942 leads to the loss of C₄ accumulation and a high-CA-requiring phenotype (Price and Badger, 1989b). Another animal-like αCA protein and gene has been discovered in *Synechococcus* PCC7942 and *Anabaena* PCC7120 (Soltes-Rak et al., 1997). This CA appears to be periplasmically located and is expressed more highly at elevated CO₂. Inactivation of the gene in *Synechococcus* PCC7942 did not impair the operation of the CCM. Thus at this stage, a carboxysomal CA protein is the only CA known to be specifically involved in the operation of the CCM.

D. Role of the Carboxysome

The carboxysomes, located within the cyanobacterial cell, have emerged to have a central role in the CCM and evidence for their functional roles has been reviewed by a number of authors over recent years (Price and Badger, 1991; Badger and Price, 1992; Kaplan et al., 1994; Price et al., 1998). These are the compartments where Rubisco and CA are specifically localized and CO₂ is elevated around the active site of Rubisco, similar in functional outcome to the C₄ bundle sheath (see Table 1). A number of mutants have been isolated and created with impaired carboxysome structure, and in all cases cells with missing or aberrant carboxysomes have a high-CO₂-requiring phenotype (see above reviews). A number of models have been developed to theoretically describe how carboxysomes might achieve their functional roles (Reinhold et al., 1989; Reinhold et al., 1991). Figure 8 summarizes one possible scenario. The key feature of this model is that the carboxysome protein shell provides a restriction to CO₂ efflux, which is around 2–3 orders of magnitude higher than that found for normal lipid bilayers such as the thylakoids and cell membrane. In addition, it must have an increased permeability for anions such as HCO₃⁻, PGA and RuBP. A conductance of around 10⁻⁴ cm s⁻¹ is sufficient to restrict CO₂ efflux and allow CO₂ to be elevated, while still allowing the exchange of HCO₃⁻, PGA and RuBP at rates sufficient to support fixation. This is because the gradients for CO₂ are around 500 μM, while for HCO₃⁻ and the sugar phosphates it will be in the 10–30 mM range. The plasma membrane is assumed to be relatively permeable to but relatively impermeable to This model assumes that Rubisco and CA are homogeneously distributed throughout the carboxysome. A more complex version of this model considers that CA molecules may be surrounded by a Rubisco shell, thus acting as a leak reduction mechanism (Reinhold et al., 1991). There is no particular evidence for this model as opposed to the simpler model shown in Fig. 8, except that one carboxysome mutant, with an extension to the C terminus of Rubisco, shows loose carboxysome structure and a high-CO₂-requiring phenotype, suggesting a role for specific Rubisco packing. Such
fine details of carboxysome structure/function remain to be resolved.

Several genes have been identified which are involved in the formation of functional carboxysomes (see Price et al., 1998, for a detailed review). Obviously the Rubisco genes and proteins are essential (rbcL and rbcS—Rubisco null mutants do not have carboxysomes—Pierce et al., 1989) and the carboxysomal CA (icfA). In addition to these a number of CCM genes and their protein products have also been identified which are involved in some aspect of the assembly of carboxysome structures. This includes ccm A, J, K, L, M, N and O (Price et al., 1998). Of these, only the ccmM appears to code for a carboxysome structural protein. This is a 58 kDa protein which at this stage is most likely a major component of the carboxysome shell. This protein possesses 3–4 internally repeated regions (depending on species) which show homology to the Rubisco small subunit. This has led to speculation that these regions may interact with Rubisco large subunit within the carboxysome in order to organize or stabilize the carboxysome structure (Price et al., 1998). The ccmK and L gene products appear to code for soluble proteins, which may be involved in assembly functions.

E. Induction of the CCM

Most cyanobacteria that have been examined show the ability to adjust the properties of their CCM, depending on C\textsubscript{i} limitation during growth. As shown in Fig. 9, cyanobacteria cannot exist without a CCM at ambient C\textsubscript{i} conditions, largely because of the low CO\textsubscript{2} affinity of their Rubisco. Cells without a CCM would require in excess of 50 mM C\textsubscript{i} at pH 8 to saturate photosynthesis. Thus a certain level of CCM is constitutively expressed in high-C\textsubscript{i} cells. However, when cells are grown at limiting C\textsubscript{i}, which may be at air or less levels of CO\textsubscript{2}, the cells develop a much higher affinity for external C\textsubscript{i}. These have been termed low-C\textsubscript{i} cells. As summarized in Table 2 certain changes occur to components of the CCM to achieve this induction. The primary change occurs to the kinetic properties of the C\textsubscript{i} transport systems, with cell increasing their affinities for CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-}. This is also accompanied by changes in the supply of energy to drive C\textsubscript{i} uptake, which may include changes to the NAD(P)H dehydrogenase complexes, as well as cyclic electron transport (see energization of C\textsubscript{i} transport section). These changes mean that cells can accumulate a larger internal HCO\textsubscript{3} pool at much lower external C\textsubscript{i} levels. There are other smaller changes which also occur to the nature of the carboxysomes, including an increase in both Rubisco and CA per cell and production of a larger number of smaller carboxysomes in the cell (Price et al., 1992; McKay et al., 1993).

The manner in which these changes are achieved is not entirely clear. Certainly some de novo protein synthesis is involved. The CmpABCD HCO\textsubscript{3} transport complex is upregulated at limiting C\textsubscript{i}, increasing the affinity of HCO\textsubscript{3} transport (Omata and Ogawa, 1986, 1987). In addition, such changes as the number and size of carboxysomes and levels of carboxysome carbonic anhydrase must also require protein synthesis changes. In general, when cells are transferred from high to low environments, adaptation to the new growth conditions takes 1–4 h and has been proposed to involve de novo protein synthesis (Omata and Ogawa, 1985; Kaplan et al., 1994). However, recently it has been shown that quite rapid induction of high-affinity HCO\textsubscript{3} transport can occur in as little as 10 min after transfer to zero CO\textsubscript{2}, in Synechococcus PCC7002 and PCC7942, and that this can be blocked by addition of protein kinase but not protein phosphatase or protein synthesis inhibitors (Sültemeyer et al., 1998a,b).
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Table 2. Adaptation of CCM components in cyanobacteria to growth at low CO₂. The CCM components are described in Fig. 3 and Table 1.

<table>
<thead>
<tr>
<th>CCM Component</th>
<th>Adaptation to growth at low C₄₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The C₄ transport system</td>
<td>The activity of both CO₂ and HCO₃-transporters are changed, resulting in an increased affinity for both external CO₂ and HCO₃. The relative contributions of HCO₃ and CO₂-transport to photosynthesis varies between species.</td>
</tr>
<tr>
<td>2. Energization of C₄ transport</td>
<td>There appears to be increased emphasis on a requirement for PS1 driven cyclic electron flow as well as the up regulation of specific NDH-dehydrogenase components, possibly located on the cytoplasmic membrane.</td>
</tr>
<tr>
<td>3. The cytosolic HCO₃ pool</td>
<td>Larger HCO₃ pools can be accumulated by low-C₄ grown cells, at lower external C₄ levels.</td>
</tr>
<tr>
<td>4. &amp; 5. The carboxysome compartment</td>
<td>A number of carboxysome changes occur. There are increases in the number of smaller carboxysomes per cell, and increases in CA and Rubisco per cell.</td>
</tr>
<tr>
<td>6. Reduction of CO₂ leakage</td>
<td>There appears to be little difference in the leakiness of low and high-C₄ grown cells, but the increased activity of the C₄ transport system may recycle CO₂ leakage at low external C₄.</td>
</tr>
</tbody>
</table>

Thus, some part of the induction process appears to be modulated by phosphorylation of existing proteins associated with a HCO₃ transport complex.

There is little evidence to suggest what components are involved in the sensing of the external C₄ environment. Some suggestions have included signal transduction pathways linked to the sensing of internal photorespiratory intermediates, external and internal C₄. Some evidence, examining rapid induction with Synechococcus PCC7002, has suggested that the external HCO₃ level may be critical to modulating the changes in phosphorylation (Sültemeyer et al., 1998a), but there may be other and multiple systems operating to control the induction of multiple CO₂ and HCO₃ transporters as well as changes to carboxysomes. Cell signal transduction in relation to adaptation to C₄ remains to be more fully explored.

V. Components and Adaptation of the CCM in Green Microalgae

A generalized description of the operation and components of the CCM in green microalgae is illustrated in Fig. 10. The salient features of this proposed model are that C₄ enters the cell either by carrier as bicarbonate or CO₂ or by diffusion as CO₂, that C₄ is transported into the chloroplast, accumulating in the stroma as bicarbonate, and that the accumulated bicarbonate enters the lumen of the pyrenoidal thylakoids where it is dehydrated by a thylakoid lumen CA (etCA1; see Figs. 10, 11 and below) to provide substrate CO₂ to Rubisco in the pyrenoid. This model of the green algal CCM is based in part on that described above for cyanobacteria and reflects primarily characteristics drawn from study of the CCM of Chlamydomonas reinhardtii. Comparison of the characteristics of this CCM to those of other CCM-systems is shown in Fig. 3 and Table 1.
A. Inorganic Carbon Transport

The requirement for active $\text{C}_4$ transport in the green-microalgal CCM was clearly established by isolation and characterization of a $\text{C. reinhardtii}$ mutant ($\text{pmp1-1}$) that lacks $\text{C}_4$ transport and accumulation (Spalding et al., 1983b), but our understanding of $\text{C}_4$ transport in microalgae remains somewhat unclear nonetheless, both in terms of location and substrate specificity. The use of carbon isotope disequilibrium studies to identify the $\text{C}_4$ species taken up has established that the major flux of $\text{C}_4$ into many green microalgae occurs through direct uptake of $\text{CO}_2$ across the plasmalemma via an active process (Sültemeyer et al., 1989; Gehl et al., 1990; Rotatore and Colman, 1991; Rotatore et al., 1992; Badger et al., 1994; Palmqvist et al., 1994b; Amoroso et al., 1998), although the data cannot distinguish between active $\text{CO}_2$ transport at the plasmalemma and active $\text{C}_4$ transport at the chloroplast envelope following $\text{CO}_2$ diffusion into the cell. From these and similar studies, there also is good evidence for direct bicarbonate transport across the plasmalemma, but generally at a lower rate than $\text{CO}_2$ influx (Sültemeyer et al., 1989; Gehl et al., 1990; Thielmann et al., 1990; Rotatore et al., 1991; Badger et al., 1994; Palmqvist et al., 1994b; Amoroso et al., 1998), except in $\text{Dunaliella tertiolecta}$, where bicarbonate appears to be the dominant inorganic carbon species taken up (Amoroso et al., 1998). Therefore, it appears that both of the predominant $\text{C}_4$ species present in the aquatic environment ($\text{CO}_2$ and $\text{HCO}_3^-$) can be used by microalgal cells, although most apparently prefer $\text{CO}_2$.

Chloroplast envelope $\text{C}_4$ transport has been demonstrated in intact, isolated chloroplasts from $\text{C. reinhardtii}$, $\text{D. tertiolecta}$, and $\text{Chlorella ellipsoidea}$, and the light-dependent $\text{C}_4$ accumulation activity of the first two was restricted to chloroplasts from air-adapted cells (Moroney et al., 1987; Sültemeyer et al., 1988; Goyal and Tolbert, 1989; Rotatore and Colman, 1990). Difficulties with obtaining high yields of intact, active chloroplasts generally have hindered extensive investigation of the $\text{C}_4$ species transported, but recent work with isolated chloroplasts from $\text{C. reinhardtii}$ and $\text{D. tertiolecta}$ indicates that both algae have inducible, high-affinity bicarbonate and $\text{CO}_2$ transporters at the chloroplast envelope (Amoroso et al., 1998).

Therefore, transport of $\text{CO}_2$ and/or bicarbonate in
microalgae may occur at the plasmalemma and/or the inner chloroplast envelope, but the mechanism of transport is not understood for uptake of either $C_i$ species across either membrane. There is evidence for the inhibition of $C_i$ uptake by the H+-ATPase inhibitor vanadate both in whole cells and chloroplasts, suggesting that activity of a vanadate-sensitive H+-ATPase is essential for transport across both the plasmalemma and the chloroplast inner envelope (Palmqvist et al., 1988; Goyal and Tolbert, 1989a; Thielmann et al., 1990; Karlsson et al., 1994). The characteristics of the C. reinhardtii pnp1-1 mutant, which completely lacks $C_i$ transport, have raised the intriguing question as to how a single mutation apparently can eliminate virtually all $C_i$ accumulation, even though there is good evidence demonstrating $C_i$ transport across both the plasmalemma and the chloroplast envelope.

Based on the available evidence, green microalgae probably have one or two plasmalemma $C_i$ transporters, one for bicarbonate and possibly another for $CO_2$. However, no plasmalemma transport proteins have been identified, nor have any proteins been identified as potential candidates for this function. The situation is similar for chloroplast envelope $C_i$ carriers, except that cDNA clones of two C. reinhardtii genes (Ccpl and Ccp2; =LIP36-G1 and LIP36-G2; see Spalding, 1998 for nomenclature) encoding closely-related, limiting-$CO_2$ inducible polypeptides showing sequence similarity with the mitochondrial carrier protein superfamily and apparently located in the chloroplast envelope have been identified recently (Ramazanov et al., 1993; Chen et al., 1997). This superfamily encompasses proteins representing a wide variety of transport substrate specificites and intracellular locations, including plastid and peroxosmal as well as mitochondrial carrier proteins (Chen et al., 1997). It is tempting to speculate that the Ccp1 and Ccp2 proteins might be involved in $C_i$ transport across the chloroplast inner envelope, but much more information is needed before the function of these proteins is established. In D. tertiolecta as well, two low $CO_2$-inducible, chloroplast envelope proteins have been reported as potential components of a $C_i$ transport mechanism (Thielmann et al., 1992). In addition, a C. reinhardtii plastid-encoded, chloroplast envelope protein (ycf10 gene product) has been implicated in $C_i$ uptake into chloroplasts (Rolland et al., 1997), although its role is unclear at present.

### B. Energization of $C_i$ Transport

The CCM requires an additional input of energy above that required for operation of the Calvin cycle, and it is clear from many early experiments that photosynthesis is the source of the additional energy (see Badger and Price, 1992). However, it is not certain how or in what form the additional energy is provided by photosynthesis. Internal $C_i$ pools are reduced or eliminated in the presence of DCMU or darkness and in the presence of proton ionophores (Badger et al., 1980; Spalding et al., 1983a; Badger, 1987). Following from this it has been proposed that a substantial part of the additional energy is required as ATP to drive active transport of $C_i$. However, considering the essential role played in C. reinhardtii by ctCA1, its probable location in the thylakoid lumen and its suggested function in the CCM (Fig. 11 and see Section V.D.), it seems apparent that at least some energy associated with generation of the thylakoid $\Delta p$H might be used to enhance the dehydration of bicarbonate in the thylakoid lumen.

Acclimation of $CO_2$-enriched C. reinhardtii and Scenedesmus obliquus to limiting $CO_2$ results in changes in the photochemical properties of the cells (Spalding et al., 1984; Palmqvist et al., 1986, 1990; Sundblad et al., 1990), indicating that the cells must adjust to the different energy demands of operating the CCM. Short term changes in the photochemical properties probably reflect the transient stress of decreased $CO_2$ availability prior to development of CCM activity, and long term photochemical changes may reflect an increased ratio of PS I to PS II to provide extra ATP to the CCM (Palmqvist et al., 1990).

### C. The Role of Carbonic Anhydrases

In recent years it has become clear that three apparently independent evolutionary lines of CA ($\alpha$, $\beta$ and $\gamma$-CA) can be found in various organisms (Hewett-Emmett and Tashian, 1996), and at least two of these lines, the $\alpha$ and $\beta$ CAs, have been found in green microalgae (Fujiwara et al., 1990; Fukuzawa et al., 1990; Hiltonen et al., 1995; Eriksson et al., 1996, Fisher et al., 1996; Funke et al. 1997; Karlsson et al., 1998). The first microalgal CA identified was the abundant periplasmic CA (pCA1) induced by limiting $CO_2$ concentrations in C. reinhardtii (Coleman and Grossman, 1984; Coleman et al., 1985).
1984), which has been studied rather extensively since its initial discovery. *C. reinhardtii* pCA1, encoded by the *Cah1* gene, is expressed to very high levels only under limiting CO$_2$ conditions, but it still is rather unclear whether it plays any essential role in the *C. reinhardtii* CCM (Williams and Turpin, 1987; Sültemeyer et al., 1990). If it does, that role may be to dehydrate bicarbonate to supply CO$_2$ for active uptake, especially under alkaline conditions (Moroney et al., 1985), a role consistent with characteristics of periplasmic CAs reported from other green microalgae (Miyachi et al., 1983; Aizawa and Miyachi, 1984; Coleman et al., 1991; Goyal et al., 1992; Pesheva et al., 1994).

Intracellular CA isozymes have long been implicated in function of the microalgal CCM, but only recently has significant progress been made in unambiguously identifying any internal CA. So far, one chloroplast-localized CA, the thylakoid lumen $\alpha$-CA (ctCA1, encoded by the *Cah3* gene) defective in mutants with lesions at the CA1 locus (Spalding et al., 1983a, Karlsson et al., 1995; 1998; Funke et al., 1997), and two mitochondrial $\beta$-CAs (mtCA1 and mtCA2, =LIP-21; encoded by the *Mca1* and *Mca2* genes; =$\beta$-CA1 and $\beta$-CA2) (Eriksson et al., 1996; Geraghty and Spalding, 1996; see Spalding, 1998 for nomenclature) have been explicitly identified in *C. reinhardtii*. Other intracellular CA isozymes have been either postulated or tentatively detected in *C. reinhardtii* (Badger and Price, 1992, 1994; Sültemeyer et al. 1993; Amoroso et al., 1996, 1998; also see Figs. 10 and 11 and Section VI.A.), but hard evidence for their existence is lacking. It is not clear what the function of the inducible mitochondrial CAs might be, but it has been suggested that they might function in pH regulation (via hydration of photorespiratory CO$_2$) to prevent alcalization of the mitochondrial matrix during rapid production of photorespiratory ammonia from the glycine decarboxylase complex and to facilitate diffusion of CO$_2$ from the same source out of the mitochondrial matrix under high photorespiratory conditions (Eriksson et al., 1996).

There is strong genetic evidence from analysis of *C. reinhardtii cal1* mutants to indicate that bicarbonate is actively accumulated in the chloroplast and that a thylakoid lumen CA (ctCA1; *Cah3* gene product) is required for rapid dehydration of this accumulated bicarbonate to supply Rubisco with CO$_2$ at a physiological rate (Spalding et al., 1983a; Suzuki and Spalding, 1989; Sültemeyer et al., 1995; Funke et al., 1997; Karlsson et al., 1998). The plastid location of ctCA1 indicates that active accumulation of bicarbonate occurs in the chloroplast, but it is not yet clear whether the accumulation occurs throughout the chloroplast or is restricted to some compartment within the plastid. It is reasonable to assume that the transported C$_i$ enters and is accumulated in the stroma as bicarbonate, regardless of which species of C$_i$ serves in the cytosol as transport substrate.

### D. The Pyrenoid as the Site of CO$_2$ Elevation

It has long been suggested that, in microalgae, the acidic thylakoid lumen might participate in net dehydration of bicarbonate to supply CO$_2$ to Rubisco at concentrations higher than the equilibrium concentration of CO$_2$ in the (presumably) alkaline pyrenoid (Pronina and Borodin, 1993; Raven, 1997), as illustrated in Figure 11. The requirements for effective participation of such a system are that the thylakoid membrane transport (or be permeable to) bicarbonate and that CA activity be present in the thylakoid lumen but not in the compartment containing Rubisco. The localization of ctCA1 to the thylakoid lumen in *C. reinhardtii* (Karlsson et al., 1998) has brought renewed interest to the possibility that such a system might operate as part of the *C. reinhardtii* CCM. Since Rubisco is localized to the pyrenoid in *C. reinhardtii* (Lacoste-Royal and Gibbs, 1987; Kuchitsu et al., 1988b, 1991; McKay and Gibbs, 1989, 1991), it is tempting to speculate that ctCA1 is restricted to those thylakoids that traverse the pyrenoid and that the high bicarbonate concentration in the stroma has access to this same pyrenoidal thylakoid lumen, thus allowing for CO$_2$ release into the pyrenoid Rubisco pool at a concentration even higher than that expected at equilibrium in the alkaline stroma/pyrenoid.

It is clear that the site of CO$_2$ elevation in *C. reinhardtii* must at least overlap with the site where bicarbonate is actively accumulated and must include both the site where the ctCA1 is located and the site where Rubisco is located. Since active bicarbonate accumulation most likely occurs in the chloroplast stroma and Rubisco appears to be exclusively located within the chloroplast pyrenoid, it has been suggested that the microalgal pyrenoid might play a role in the CCM similar to the role apparently played by the carboxysome in cyanobacteria (Badger and Price, 1992,1994). Depending on whether ctCA1 is located in all thylakoids or...
restricted to those encompassed by the pyrenoid, the site of CO₂ elevation might be restricted to the pyrenoid or include the whole chloroplast. In this regard, it is intriguing to note that at least one alga that lacks a pyrenoid has also been found to lack a functional CCM (Palmqvist et al., 1994a, 1995).

In keeping with the hypothesis that the site of CO₂ release from actively accumulated bicarbonate might be restricted to the pyrenoid, it has been suggested that the starch sheath might serve as a diffusion barrier to minimize loss of CO₂ from the pyrenoid and thus from the site of Rubisco activity (Badger and Price, 1992, 1994). However, recent work with mutants of both C. reinhardtii and Chlorella pyrenoidosa lacking pyrenoid starch sheaths showed that the affinity of the mutants for CO₂ in photosynthesis was not significantly different from that of wild-type cells (Plumed et al., 1996; Villarejo et al., 1996a). Thus any role as a diffusion barrier played by the pyrenoid starch sheath must be minimal at best. Since the chloroplast envelopes probably do not represent a significant diffusion barrier to CO₂, the existence of any barrier to restrict diffusion of the elevated CO₂ away from its source, whether that be the pyrenoid or the whole chloroplast, is still in question.

**E. Induction of the CCM and Related Adaptations to Limiting CO₂**

Green microalgae can exist in at least two distinctly different physiological states depending on the CO₂ concentration (0.03% vs 1–5%) during growth (see Spalding, 1989), which reflects a whole suite of adaptive responses to limiting CO₂ (Table 3). Acclimation to limiting CO₂ in C. reinhardtii results in, along with induction of a functional CCM, the induced expression of specific genes, including Cah1, Ccp1 and Ccp2, and Mca1 and Mca2, encoding the major periplasmic CA (pCA1), the putative chloroplast carrier proteins (Ccp1 and Ccp2) and the mitochondrial CAs (mtCA1 and mtCA2), respectively (Coleman and Grossman, 1984; Bailly and Coleman, 1988; Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Fujiwara et al., 1990; Fukuzawa et al., 1990; Geraghty et al., 1990; Spalding et al., 1991; Ramazanov et al., 1993; Eriksson et al., 1996; Geraghty and Spalding, 1996; Chen et al., 1997). In addition to the identified genes, two soluble polypeptides of 45–50 kDa appear to be up-regulated (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989), and substantial increases also occur in the activity of photorespiratory enzymes (Marek and Spalding, 1991; Ramazanov and Cardenas, 1994). Two limiting-CO₂-induced polypeptides also have been reported in D. tertiolecta chloroplast envelopes (Thielmann et al., 1992).

Six C. reinhardtii cDNA clones of other limiting-CO₂-inducible genes were reported by Burow et al. (1996), one of which corresponded to the Cah1 gene. The sequence of one, Lecl1, showed no homology to any known genes, but its deduced amino acid sequence included four putative transmembrane spanning helices, suggesting it might encode a transmembrane protein. Another of the clones (Att1) was subsequently identified as encoding alanine: α-ketoglutamate aminotransferase, although it is not clear what role this aminotransferase might play in acclimation of C. reinhardtii to limiting CO₂ (Chen et al., 1996).

During acclimation of C. reinhardtii to limiting CO₂, expression of Cah1, Mca1, Mca2, Ccp1 and

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**Table 3. Adaptation of carbon concentrating mechanism (CCM) components in green algae to growth at low CO₂. The CCM components are described in Fig. 3 and Table 1.**

<table>
<thead>
<tr>
<th>CCM Component</th>
<th>Adaptation to Growth at Low CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. The C₅ transport system</strong></td>
<td>C₅ transport, either as CO₂ or HCO₃⁻, increases from little or no activity prior to adaptation both at cell and chloroplast membranes. The relative contributions of CO₂ and HCO₃⁻-transport are unclear at both locations.</td>
</tr>
<tr>
<td><strong>2. Energization of C₅ transport</strong></td>
<td>Although changes occur in the photochemical properties of the cells during adaptation, it is not yet clear how these are related to the increased energy demands associated with operation of the CCM.</td>
</tr>
<tr>
<td><strong>3. The stromal HCO₃⁻ pool</strong></td>
<td>No significant HCO₃⁻ pool can be detected prior to adaptation.</td>
</tr>
<tr>
<td><strong>4. The thylakoid lumen CA</strong></td>
<td>The significant changes appear to occur with regard to the thylakoid lumen CA.</td>
</tr>
<tr>
<td><strong>5 &amp; 6. The pyrenoid compartment</strong></td>
<td>It is unclear whether any changes occur in the pyrenoid itself. The pyrenoid starch sheath increases, but it is not clear whether this is of any functional significance for the CCM.</td>
</tr>
</tbody>
</table>
Ccp2 all are regulated at the level of mRNA abundance and appear to be closely correlated (Bailly and Coleman, 1988; Dionisio-Sese et al., 1990; Fujiwara et al., 1990; Geraghty et al., 1990; Spalding et al., 1991; Eriksson et al., 1996, 1998; Geraghty and Spalding, 1996; Chen et al., 1997), suggesting that their expression may be coordinately regulated by a single mechanism, e.g., a single trans-acting factor. Along with the genes induced in limiting \( \text{CO}_2 \), some genes exhibit decreased expression in limiting \( \text{CO}_2 \), including stable down-regulation of the minor periplasmic CA (\( \text{Cah}2 \)) (Fujiwara et al., 1990) and transient decrease in the synthesis of both subunits of Rubisco (the \( \text{Rbc}S1, \text{Rbc}S2 \) and \( \text{rbc}L \) gene products) (Coleman and Grossman, 1984; Winder et al., 1992). Although \( \text{Cah}2 \) expression is controlled at the level of mRNA abundance, as with the five induced genes discussed above (Fujiwara et al., 1990; Rawat and Moroney, 1991), the transient decrease in biosynthesis of both Rubisco subunits during acclimation to limiting \( \text{CO}_2 \) is controlled at the translational level (Winder et al., 1992).

Acclimation to limiting \( \text{CO}_2 \) also can involve substantial structural changes in microalgal cells, including an increased development of the pyrenoid starch sheath (Kuchitsu et al., 1988a, 1991; Ramazanov et al., 1994; Geraghty and Spalding, 1996), increased vacuolization (Geraghty and Spalding, 1996) and changes in mitochondrial distribution (Kramer and Findenegg, 1978; Geraghty and Spalding, 1996). Mitochondria of \( \text{C. reinhardtii} \) move during acclimation from a central position, within the cup of the chloroplast, to a peripheral position, between the chloroplast envelope and the plasma-lemma (Geraghty and Spalding, 1996).

The mitochondrial relocation and the limiting-\( \text{CO}_2 \) induction of specific mitochondrial proteins (Eriksson et al., 1996; Geraghty and Spalding, 1996) argue that the mitochondria may play an important role in acclimation of green microalgae to limiting \( \text{CO}_2 \). It has been suggested that, because of their move to a peripheral location in air-adapted cells and the increased glycolate pathway flux in air-adapted cells, the mitochondria might be involved in scavenging a photorespiratory metabolite produced by the chloroplasts (Geraghty and Spalding, 1996).

The signal regulating acclimation of green microalgae to changes in \( \text{CO}_2 \) concentration is unknown (Coleman, 1991). Clearly, they must sense the change either directly as \( \text{CO}_2 \) (or \( \text{C}_2 \)) concentration or by an indirect effect on a cellular process, such as carbohydrate metabolism. Several studies have implicated a requirement for photosynthetic activity for induction in \( \text{C. reinhardtii} \) (Spalding and Ogren, 1982; Spencer et al., 1983; Dionisio et al., 1989a,b; Dionisio-Sese et al., 1990; Villarejo et al., 1996b). Based on evidence implicating photorespiration in induction, it has been suggested that a \( \text{CO}_2 \) deficiency might be signaled by a photorespiratory metabolite (Spalding and Ogren, 1982; Ramazanov and Cardenas, 1992; Villarejo et al., 1996b). However, this simplistic model fails to take into account reports indicating a more complicated signaling system, including induction by low \( \text{CO}_2 \) concentrations in the dark (Bailey and Coleman, 1988; Fett and Coleman, 1994; Rawat and Moroney, 1995; Villarejo et al., 1996b), enhanced induction by non-photo-synthetic blue light (Dionisio et al., 1989a,b; Dionisio-Sese et al., 1990; Borodin et al., 1994) and partial repression by mixotrophic growth with acetate as a carbon source (Spalding and Ogren, 1982; Moroney et al., 1987; Coleman et al., 1991; Fett and Coleman, 1994; Ramazanov et al., 1994). Clearly the acclimation of \( \text{C. reinhardtii} \) to limiting \( \text{CO}_2 \) involves a signaling system more complex than a change in the concentration of a photorespiratory metabolite, but, at present, it is difficult to incorporate all the available information on \( \text{Cah}1 \) and CCM induction into a cogent hypothesis for the signaling mechanism.

An important advance in understanding gene regulation mediated in \( \text{C. reinhardtii} \) by changes in \( \text{CO}_2 \) concentration was made with the identification and characterization of a mutant, cia5, which apparently does not acclimate to limiting \( \text{CO}_2 \) (Moroney et al., 1989). The cia5 mutant lacks induction of \( \text{C}_2 \) transport, induction of \( \text{Cah}1, \text{Mca}1 \) and \( \text{Mca}2, \text{Ccp}1 \) and \( \text{Ccp}2, \text{Lci}1 \), or any of the unidentified limiting-\( \text{CO}_2 \)-induced polypeptides, up-regulation of phosphoglycolate phosphatase and glycylate dehydrogenase, and down-regulation of Rubisco biosynthesis (Moroney et al., 1989; Marek and Spalding, 1991; Spalding et al., 1991; Burow et al., 1996). The lack of any of these responses in cia5 argues that one signal transduction pathway regulates a very diverse set of adaptations, including transcriptional (pre-translational) up-regulation of \( \text{Lci}1, \text{Cah}1, \text{Mca}1, \text{Mca}2, \text{Ccp}1 \) and \( \text{Ccp}2 \), transcriptional (pre-translational) down-regulation of \( \text{Cah}2 \), transient, translational down-regulation of \( \text{rbc}L \) (chloroplast), \( \text{Rbc}S1 \) (nuclear) and \( \text{Rbc}S2 \) (nuclear) expression, long term up-regulation of glycylate dehydrogenase and transient up-regulation of phosphoglycolate phosphatase. Since cia5 appears to lack any response to limiting \( \text{CO}_2 \), this mutant
probably represents a defect in a component of the signal transduction pathway that is required for induction of all of the observed limiting CO₂ adaptations.

VI. Diversity of CCM Function in Green and Non-Green Algae

A. Potential Diversity of CCMs in Algae

The existence of CCMs and their contribution to aquatic photosynthesis is well recognized (Aizawa and Miyachi, 1986; Badger, 1987; Spalding, 1989; Kaplan et al., 1991; Badger and Price, 1992). However, most of the studies of these systems have centered on model cyanobacterial and green microalgal systems. However, for algae in particular it is likely that considerable diversity in the operation of CCMs exists between genera belonging to both green and non-green algae. In this context, more detailed studies of algal species across all the algal groups are necessary before we can understand the range of solutions which have been found to circumvent Rubisco’s limitations.

In considering the diversity of CCM function in both green and non-green algae it is useful to consider the possible functional elements which a eukaryotic cell may possess to enable an elevation of CO₂ within the chloroplast. In this context, consideration of Table 3 and Fig. 10 for Chlamydomonas is useful. These elements include:

1. One or more plasma membrane located C₄ transport systems.
2. The presence of various forms of external periplasmic carbonic anhydrase.
3. One or more chloroplast membrane-located C₄ transport systems which use photosynthetic energy to accumulate HCO₃⁻ within the chloroplast.
4. Pyrenoid or stromal Rubisco, which may be an integral part of CCM activity.
5. A pyrenoid carbonic anhydrase.
6. A thylakoid CA which might use luminal protons to generate CO₂ either in the pyrenoid or the stroma surrounding the thylakoids.
7. An envelope CA which could minimize CO₂ leakage by converting CO₂ back to HCO₃⁻.
8. The possible significance of single versus multi-chloroplast cells.
9. Variation in the kinetic properties of Rubisco.
10. Involvement of mitochondria, including mitochondrial CA.

Although the above list includes most known possible elements of an algal CCM, not all elements would be expected in all algae displaying CCM ability. It is possible to envisage a number of CCM arrangements, scaling from those that perhaps have no CCM at all, to algae where the maximum amount of CCM activity is engaged. However, all algal CCM models which have been thus far described (Spalding and Portis, 1985; Fridlyand, 1997; Raven, 1997; Badger et al., 1998; Spalding, 1998) have one property in common, that being the active accumulation of C₄, resulting in the accumulation of HCO₃⁻ in the chloroplast stroma. This HCO₃⁻ supply can be used in a number of ways to elevate CO₂, through the action of CA located in three possible places, the pyrenoid, the thylakoid lumen or the stroma (Badger et al., 1998).

B. Indications of CCM Diversity between Species

The assessment of the diversity of CCM functioning and activities across diverse microphyte and macrophyte algal genera depends on the availability of reliable indicators for measuring and quantifying the relative functioning of a CCM. A survey of the experimental evidence that has been accumulated for a range of algal species has recently been presented (Badger et al., 1998) and suggests that the following list of experimental indicators may be used to measure aspects of CCM activity. Table 4 shows a summary of both green and non-green algal species that have been examined for various CCM attributes and summarizes some general findings with regard to diversity. Aspects of this diversity are expanded in the following sections.

1. C₄ Accumulation

Since the discovery of a CCM in both Chlamydomonas reinhardtii and Anabaena variabilis (Badger
et al., 1980; Kaplan et al., 1980) it has generally been recognized that there needs to be light-dependent active accumulation of an internal \( C_i \) pool for a CCM to function. The physical existence of this has been measured by both silicone-oil centrifugation techniques and by measuring uptake and evolution during dark/light/dark transients (Badger et al., 1980; Badger et al., 1985; Miller et al., 1991). While this has proved relatively easy with cyanobacteria, because of their large internal pools, evidence is much less reliably collected for algae which have smaller accumulation ratios (Burns and Beardall, 1987). In addition, these techniques do not work well with macroalgae which are not amenable to some of these physical separation techniques. As has been pointed out by Raven (1997) and by Badger et al. (1998), the expected internal \( C_i \) pool may be very dependent on what particular CCM might operate. Certainly the involvement of the thylakoid proton-linked CA may reduce pools, and actually lead to a depletion of total \( C_i \) but an accumulation of \( CO_2 \). Thus, at this stage, while measurement of a \( C_i \) pool is useful in establishing that this is part of a particular CCM, the absence of significant accumulation does not mean the absence of a CCM. Indeed, it may be useful to establish in how many species \( C_i \) accumulation or
non-accumulation is a characteristic of a CCM.

Examining the available data (Badger et al., 1998), it is apparent that it is really only the green microalgae such as Chlamydomonas, Dunaliella and Chlorella which show what can be called significant levels of C₄ accumulation in the cells. Non-green microalgae which have been measured have significantly lower levels of C₄ accumulation, despite the fact that many of them can achieve similarly high affinities for external C₄, particularly at the alkaline pH of seawater. Examples of this include Phaeodactylum, Isochrysis and Porphyridium. Chondrus crispus is also an example of a red macroalga with an absence of apparent C₄ accumulation but with a relatively high photosynthetic affinity for external C₄. Exceptions in the non-green algae appear to be the freshwater diatom, Navicula pelliculosa and the acid thermophile Rhodophyte Cyanidioschyzon merolae, which both show significant C₄ accumulation. Significant C₄ accumulation also appears to occur in the dinoflagellate Peridinium gatunense.

2. CO₂ Affinity of Photosynthesis Versus Rubisco

The driving force for the evolution of a CCM has been the low affinity of Rubisco for external CO₂. It is not surprising then that one of the most useful indicators for implying the existence of a CCM in any photosynthetic organism is to compare the affinity of photosynthesis for external CO₂ versus the affinity of its Rubisco. The major limitation of this approach is the ability to accurately measure the affinity of Rubisco extracted from various organisms that may be more or less recalcitrant to in vitro measurement techniques.

Using this comparative approach, it is relatively easy to conclude that Chlamydomonas reinhardtii with a $K_m$(CO₂-photosynthesis): $K_m$(CO₂-Rubisco) of about 1:30, clearly employs a CCM to improve Rubisco kinetics (see Badger et al., 1998). In contrast, Coccomyxa with a ratio of about 1:1 appears not to employ a CCM. A high photosynthetic affinity of intact cells for CO₂ compared to Rubisco exists in wide range of green and non-green algae, particularly microalgal species, achieving ratios of greater than 2:30 in many cases. The notable exceptions appear to be the microalgal species Coccomyxa, Emiliania huxleyi and Gonniotrichopsis sublittoralis, as well as the macroalgae Palmaria palmata and Laminaria species. Considering the dinoflagellates, which possess a Form II Rubisco (see Fig. 4), one would also expect a relatively efficient CCM to achieve high affinities for external CO₂ despite a Rubisco which has a relatively low $S_{1/2}$ and probably a high $K_m$(CO₂).

3. Oxygen Inhibition of Photosynthesis and the CO₂ (C₄) Compensation Point

Based on the functioning of Rubisco in higher plants and the central role of its oxygenase reaction in the photorespiratory cycle, evidence for oxygen inhibition of photosynthesis in algae and the CO₂ compensation point has been used to infer some potential for CCM activity. The obvious assumption in this approach is that algal Rubisco enzymes are similar to those of higher plants and that when CO₂ is subsaturating, then atmospheric levels of oxygen would be expected to inhibit carboxylation and enhance the synthesis of phosphoglycolate. Interestingly, for most algal and cyanobacterial species, except some green algae, the effects of oxygen on photosynthesis are significantly less than that which would be expected in higher plants and could lead to the inference that CCM activity is present to suppress oxygenase activity, as occurs in C₄ higher plants (Kerby and Raven, 1985; Burns and Beardall, 1987; Beardall, 1989; Spalding, 1989; Raven et al., 1990; Johnston, 1991). With an increase in our knowledge of the kinetics of Rubisco enzymes from a variety of algal and cyanobacterial sources it is apparent that this approach may be of little use for most algal species, particularly non-green algae and cyanobacteria.

4. Carbonic Anhydrases

As CA is of central importance to all CCM models shown in Fig. 10, then examining the presence of both internal and external CAs and the effects of CA inhibitors has proven useful for implying the function of a CCM in algae.

External CA has been readily measured in a wide range of species and has been well correlated with the utilization of external HCO₃⁻. External CA has been shown to function in both micro and macroalgae in all algal Divisions (Burns and Beardall, 1987; Giordano and Maberly, 1989; Johnston, 1991; Badger and Price, 1992).

Internal CAs have been less well studied in non-green algae. A particular exception to this has been
chloroplast CA from Porphyridium (Mitsuhashi, personal communication) where recent evidence suggests that this may be a soluble protein localized in the matrix of the Rubisco containing pyrenoid, rather than being associated with the thylakoid as has been discovered for Chlamydomonas (Karlsson et al., 1998). The green alga Coccomyxa (Hiltonen et al., 1995), which lacks a pyrenoid, has also been shown to possess a soluble β-type CA which is presumed to be localized in the stroma. Thus we may expect the further discovery of multiple forms of internal CA with multiple locations and particular roles in a CCM. Of particular recent interest has been the description of a carbonic anhydrase gene from the oceanic diatom Thalasiosira weissflogii (Roberts et al., 1997) which shows little homology to other CA genes and proteins from either cyanobacteria or green algae.

5. Light-Stimulated CA Activity

A primary component of all CCMs is that light energizes the uptake of external inorganic carbon and places it in an internal environment containing CA. This constitutes a light-stimulated CA activity. Such light-stimulated CA activity is only measurable with a mass spectrometric approach, examining the exchange of 18O from C₅ species into 16O labeled water. This has been used to show light-stimulated CA activity in both cyanobacteria (Badger and Price, 1989) and green algae (Palmqvist et al., 1994b) and non-green algae (Badger et al., 1998) which employ a CCM. It is apparent that there is considerable diversity in the appearance of light-stimulated CA among the non-green algae which remains to be explored and explained.

6. The Use of HCO₃⁻ as a Photosynthetic Substrate

Bicarbonate usage in photosynthesis has long been examined as a property of algae (Lucas, 1983). Usage of HCO₃⁻ by algae is obviously dependent on the participation of external CA and plasma membrane transporter(s) (Badger, 1987; Johnston, 1991; Badger and Price, 1992). In all algae so far examined, it would appear as though HCO₃⁻ usage is well correlated with the presence of a CCM (see Badger et al., 1998). The green algae Stichococcus bacillaris and Chlorella saccharophila and the Rhodophyte Cyanidioschyzon merolae are exceptions, largely using CO₂ but showing clear evidence of CCM activity.

7. Changes in the Affinity for External C₅, Depending on Growth Conditions

The induction of a high affinity for external C₅ when cells are grown at limiting C₅ is well recognized as being due to the induction of CCM activity in microalgae (Badger, 1987). Changes in the affinity for external C₅ of 10 fold or more are well documented in both green and non-green algae (Badger et al., 1998). Such changes have been shown to involve changes in the affinity of C₅ transport systems for external CO₂ and HCO₃⁻ as well as other changes involving increases in both internal and external CA activities (section V.D). Such inducible changes seem to be strong evidence of the participation of cellular infrastructure in the supply of CO₂ to Rubisco in the chloroplast and are easily measured by examining photosynthesis in intact cells. These inducible changes are not limited to microalgae and have also been observed in a number of macrophytes, including Ulva (Bjork et al., 1993), Gracilaria (Garcia Sanchez et al., 1994), Porphyra (Mercado et al., 1997) and Fucus (Johnston and Raven, 1990). While such changes indicate an active supply of CO₂ to Rubisco, particularly when coupled with HCO₃⁻ usage, they do not allow a distinction to be made as to whether the changes occur to processes involving the chloroplast or the cytosol/plasma membrane components of the cell. The lack of inducible changes in affinity for external C₅ is certainly not evidence for the lack of a CCM. Inducible changes in a CCM probably only occur in species that experience periodic limitations to external C₅ supply. Many algal species may employ a CCM with variable activity that is sufficient to meet their environmental needs. They may not have the very high affinities for external C₅ exhibited by some microalgae but are nonetheless dependent on the engagement of some level of CCM.

8. Correlations between Pyrenoids, Plastid Number and CCMs

The role of the Rubisco containing pyrenoids in the algal CCM has been of considerable speculation since the identification of the cyanobacterial carboxysome as central to the cyanobacterial CCM (Badger and Price, 1992). But, speculation and correlation is all that it has remained. Certainly,
Rubisco has been found to be localized within a number of green and non-green algal pyrenoids (Lacoste-Royal and Gibbs, 1987; Kuchitsu et al., 1988b; McKay and Gibbs, 1989, 1991) and CA has been found in the pyrenoid of the red alga Porphyridium (Mitsuhashi, personal communication).

So far we have four examples where the absence of pyrenoids seems to be correlated with the absence or reduction in CCM activity. There are three algae, Coccomyxa (Palmqvist et al., 1994a, 1995), Trentopohlia (Smith and Griffiths, 1996a), Goniottrichopsis (Badger et al., 1998) and one bryophyte, Anthoceros crispulus (Smith and Griffiths, 1996a,b) as examples where the absence of CCM activity appears to be correlated with an absence of pyrenoids in the chloroplast. In Trentopohlia, Goniottrichopsis and Anthoceros, this is also correlated with multi-plastid cells. Pyrenoids abound in all algal Divisions and the evolution of more advanced genera in all algal groups is associated with the loss of pyrenoids and a transition from large single plastids to smaller plastids and multiplastidic cells. Badger et al. (1998), have summarized data which show that there are plentiful examples of closely related algal genera showing variations in both the presence and absence of pyrenoids as well as the number of plastids.

Investigation of the pyrenoid literature (Griffiths, 1970; Dodge, 1973; Ettl, 1978,1983; Garbary et al., 1980; Bold and Wynne, 1985; McKay and Gibbs, 1991) shows that there is a wide diversity in pyrenoid structure and morphology. The simplest pyrenoids consist mainly of a protein matrix containing Rubisco and with no chloroplast thylakoids entering this body. In more complicated pyrenoids, thylakoids enter the protein matrix in either a random or more organized arrangement. Mostly, pyrenoid bodies are contained within the general stromal compartment of the chloroplast but in some cases the pyrenoid is stalked, protruding outside the body of the chloroplast, often into the interior of the cell. In addition to this, starch and polysaccharide may be associated with pyrenoids, normally surrounding the pyrenoid as a sheath. There may also be one or more pyrenoids per chloroplast. There has been some speculation as to the role of various pyrenoid features, particularly as they may be related to CCM function. Studies of pyrenoid-located thylakoids have indicated that there may be a dominance of PS I in these lamellae and they may be involved in cyclic electron transport (McKay and Gibbs, 1991). It is tempting to speculate that cyclic electron transport may be involved in the supply of protons which may aid in the conversion of HCO₃⁻ to CO₂, in the mechanism depicted in Fig. 11, but there is no evidence to support this notion. Pyrenoid structure has strong phylogenetic linkages in all algal groups and has been used by many as an aid in algal classification. The unique structure/function relationships of pyrenoids, particularly in their role in the chloroplast CCM, remain to be fully understood.

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Chapter 16  

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Chapter 17

Photosynthetic Fractionation of Carbon Isotopes

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Summary

During photosynthetic CO₂ fixation fractionation of stable carbon isotopes occurs and, consequently, plants are generally depleted in the heavier isotope ¹³C. Carbon isotope discrimination (Δ) is a measure of this process and depends on fractionation during diffusion and during enzymatic carboxylation reactions. Discrimination during photosynthesis has a significant, though relatively small, effect on the isotopic composition of atmospheric CO₂ both at regional and global level; hence stable isotopes find relevant applications in the study of the global carbon cycle. In addition to variation in Δ among plants with different photosynthetic pathways, large variations are found within plant groups, resulting from genetic and environmental influences on the ratio of partial pressures of CO₂ at the sites of carboxylation and that in the free turbulent atmosphere. Experimental evidences confirming the theory of carbon isotope discrimination and known complications are discussed. Carbon isotope composition also varies among different metabolites, compartments and plant organs as a result of fractionation during secondary metabolism and variation in the ratio of diffusional and carboxylation limitations. Special emphases are given to measurements of Δ in different carbon pools such as bulk dry matter, cellulose, starch and sucrose, with different turnover rates and different integration of ¹³C and to the links with water-use efficiency. The application of carbon isotope discrimination to physiological and ecophysiological studies and to selection of genotypes with improved water-use efficiency and drought tolerance and the recent progress in this field are reviewed.

I. Introduction

In nature there are two stable isotopes of carbon, ¹²C and ¹³C. The lighter isotope ¹²C is by far the most abundant with about 98.9% of the atoms, while the heavier isotope ¹³C is present in variable proportion at around 1.1%. These two isotopes are not equally distributed in natural compounds because of isotope fractionation occurring during physical, chemical and biological processes involved in the carbon cycle. Normally plants are depleted in ¹³C compared to atmospheric CO₂ because of carbon isotope fractionation occurring during photosynthetic CO₂ fixation.

The interest in stable isotope distribution in nature developed initially in the physical sciences and then became a focal point for geochemists. Indeed, the earliest contributions to botany with measurements of plant isotopic compositions were by geochemists interested in natural variations in isotope abundance levels. Since the early systematic measurements of carbon isotope composition in plants (Wickman, 1952; Craig, 1953) there have been numerous efforts to understand the mechanisms determining the isotope fractionation during photosynthesis (Park and Epstein, 1960, 1961). Early systematic surveys of carbon isotope ratios in plants possessing the C₃ and the C₄ photosynthetic pathways were reported by Margaret Bender (1968, 1971) and Smith and Epstein (1971), shortly after the discovery of the C₄ photosynthetic pathway (Kortschak et al., 1965; Hatch and Slack, 1966). Then, carbon isotope abundance become a classical means to distinguish between different photosynthetic pathways and to study their geographical, taxonomic and ecological distributions. Subsequently, several mechanistic models were developed to explain the fractionation in C₃ and C₄ plants and to account for the variability in isotope composition observed among plants within each photosynthetic pathway. On the basis of these models, a large number of investigations have focused on the study of the relationship between carbon isotope fractionation and water-use efficiency, especially in C₄ species. The enormous success of these studies has allowed the application of stable isotopes in genetic studies and in breeding program for increased yield in dry environments.

The bases of carbon isotope fractionation in plants have been previously reviewed (O’Leary, 1981; Peterson and Fry, 1987; O’Leary, 1988; Farquhar et
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al., 1989a). However, more recently a large number of studies has extended the knowledge of carbon isotope discrimination during photosynthesis.

In this chapter, the fundamental chemical, physical and biochemical principles of carbon isotope fractionation in plants are reviewed. The effects of fractionation processes occurring during photosynthesis and during carbon metabolism on plant isotope composition are discussed. Special attention is also given to recent developments in this area and to possible implications for physiological and ecological studies of photosynthesis and plant productivity. Perspectives of stable isotopes in the study of water-use efficiency and productivity and the application in breeding for increased yield and drought tolerance are also discussed.

II. Carbon Isotopes in Nature—The Global Carbon Cycle

Variation of carbon isotope composition occurs in the organic and inorganic carbon pools. Atmospheric carbon dioxide represents the major link between the inorganic and organic portions of the terrestrial global carbon cycle and between terrestrial and marine ecosystems (Siegenthaler and Sarmiento, 1993). It participates in the equilibrium exchange reactions between the ocean carbonates and serves as the major source of carbon for the entire biosphere, as a substrate for photosynthesis.

Apart from carbon in the earth’s crust, dissolved inorganic carbon in the oceans is the largest pool in the global carbon cycle. It is mostly represented by HCO₃⁻, but CO₃²⁻ and dissolved CO₂ are also present. The isotope ratio of total dissolved inorganic carbon and of marine carbonates are both close to 0‰ (Bauer et al., 1995) relative to the international standard PDB (Pee Dee Belemnite, see below), which is also a carbonate limestone of marine origin. Because of photosynthetic fractionation, the organic carbon pool in the marine environment is strongly depleted in ¹³C compared to the inorganic carbon pool. Dissolved organic carbon is mostly represented by soluble products derived from decomposition of plankton, and is isotopically lighter (about –22‰ compared to PDB) than dissolved inorganic carbon (Boutton, 1991; Bauer et al., 1995).

Since the ocean, and particularly its dissolved inorganic carbon, is a much larger carbon pool than the atmosphere, the isotope ratio of atmospheric CO₂ is largely determined by the ocean-atmosphere CO₂ exchange together with fractionation during the CO₂ exchange with the terrestrial biosphere by photosynthesis and respiration. The isotopic composition of CO₂ would be, at equilibrium, depleted by about 7‰ in ¹³C compared to the total inorganic pool dissolved in the surface ocean water (Mook, 1986). The isotope composition of atmospheric CO₂ varies seasonally, anti-parallel with fluctuation in CO₂ concentration, as a consequence of variation in photosynthesis and respiration (Keeling et al., 1996). In the Northern hemisphere, this fluctuation has a maximum in autumn and a minimum in spring (Mook et al., 1983). In the Southern hemisphere variations in atmospheric CO₂ isotope ratio are less pronounced because of greater activity in the tropics where the seasonality is less marked, and six months out of phase compared to the Northern hemisphere.

The carbon isotope ratio of atmospheric CO₂ is also subjected to a long-term decline associated with the increase in CO₂ concentration due to anthropogenic fossil fuel combustion and deforestation (Keeling et al., 1979; Mook et al., 1983; Francey, 1985; Friedli et al., 1986, 1987). The isotope composition of the CO₂ produced from human activities is invariably depleted in ¹³C, compared to the atmospheric carbon isotope ratio. Consequently, fossil fuel combustion and deforestation result in the progressive dilution of the ¹³C content of the atmosphere.

Atmospheric methane and carbon monoxide represent much smaller carbon pools than CO₂. The atmospheric concentration of CH₄ has more than doubled in the past 200 years with a strong covariation of CO₂ and CH₄ concentrations (Battle et al., 1996). Changes in concentrations are reflected in a moderate increase of the ¹³C content of CH₄, with δ being presently around –48‰ (Craig et al., 1988; Lowe et al., 1993). Sources, sinks and fluxes of atmospheric CO are poorly known. Concentrations and isotope compositions of CO are highly variable; nevertheless the global CO concentration increased during the 1980s because of human activities (car exhaust, agricultural waste and burning of savanna). Recently, it has been reported that global CO concentration has started to decline, possibly because of a reduction in emissions (Khalil and Rasmussen, 1994). Moderate decreases in emission would be reflected in a decreased global CO concentration because of its short atmospheric lifetime (2–3 months). Oxidation of CH₄ and CO by atmospheric chemistry produces
CO₂ depleted in ¹³C. Hence the effect on the isotopic composition of atmospheric CO₂, although small, would be toward more negative δ values.

After photosynthetic CO₂ fixation by autotrophic organisms carbon is then cycled through heterotrophic organisms and detritus. During this trophic cycling the isotope ratios of carbon are maintained (Gearing, 1991). This is the meaning of the saying that, ‘You are what you eat,’ although a trend toward enrichment in ¹³C with increasing trophic levels of about 1‰ per level does exist (De Niro and Epstein, 1978). Hence, the isotope composition of the entire biosphere is largely determined by the fractionation processes occurring during photosynthesis.

III. Definitions

A. Isotope Composition

Carbon isotope composition is generally measured on CO₂ by isotope ratio mass spectrometers. The material of interest is converted to CO₂ and then injected into the inlet of the mass spectrometer. Plant material is usually converted to CO₂ by combustion, and increasingly, using elemental analyzers. The latter are often coupled to mass spectrometers for automated combustion and analysis. Mass spectrometric analysis gives high precision measurements of the isotope abundance ratio R, defined as

\[ R = \frac{^{13}C}{^{12}C} \]  

However, absolute isotope composition is not easily measured directly, and mass spectrometers measure the deviation of the sample isotope ratio from that of a known standard. Hence the isotope composition δ¹³C is defined by:

\[ \delta^{13}C = R_p/R_s - 1 \]  

where \( R_p \) is the isotope ratio in plant samples and \( R_s \) is the ratio of the standard. The internationally accepted standard for carbon isotope measurements is CO₂ produced from a Cretaceous belemnite from the Pee Dee formation in South Carolina, and referred to as PDB. Early measurements of the isotope ratio of PDB indicated a value of 0.01124 (Craig, 1957), while recent analysis suggests that a value of 0.01118 is more appropriate (Bakke et al., 1991). Isotope composition is usually referred relative to PDB, with the deviation often conveniently expressed as parts per thousand (i.e., \( \times 10^{-3} \) or ‰, ‘per mil’). However, the supply of PDB standard has been exhausted and, consequently, the Commission on Atomic Weights and Isotopic Abundances of the International Union of Pure and Applied Chemistry has recently recommended (Coplen, 1995) that isotopic abundances for carbon be reported relative to VPDB (Vienna Pee Dee Belemnite). This is defined by adopting a δ¹³C value of +1.95‰ for NBS 19 carbonate relative to VPDB. Nevertheless, PDB is currently the widely adopted standard in studies of carbon isotope composition.

B. Isotope Effects

Variation of isotope ratios and isotope compositions among different compounds is due to isotope fractionation during physical, chemical and biological processes. The isotope fractionations during such processes are described as isotope effects, the ratio of the rate constants for reactions of different isotopic species. Isotope effects are often divided into kinetic isotope effects and thermodynamic or equilibrium isotope effects. Kinetic effects are those in which a substrate or source is converted into a product with fractionation against the heavier isotope and, therefore, the product is isotopically lighter than the substrate. The kinetic isotope effect can be described as the ratio of the rate constants for the substrate containing and and when the source is a reservoir large enough to be not appreciably affected by product formation, then the isotope effect, \( \alpha_k \), is equal to the ratio of isotope ratios of source and product:

\[ \alpha_k = k^{12}/k^{13} = R_s/R_p \]  

where \( R_s \) and \( R_p \) are the isotope ratios of the source and of the product, respectively. Similarly, the equilibrium isotope effect is defined as the ratio of the equilibrium constants \( K_r^{12} \) and \( K_r^{13} \), for the molecules containing ¹²C and ¹³C, respectively.

Because equilibrium isotope effects are the resultant of opposing kinetic isotope effects, the kinetic isotope effects are generally larger than equilibrium ones. Fractionation associated with ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is a kinetic isotope effect. Another kinetic isotope effect is represented by the difference in the binary diffusivities of ¹²CO₂-air and ¹³CO₂-air, with
12CO2 diffusing faster in air than the heavier isotopic species. Examples of equilibrium isotope effects are the fractionation between CO2 in air and in solution, and the effect associated with hydration of CO2 to HCO3. It is important to note that in a multistep fractionation sequence, equilibrium effects are additive while kinetic effects are not (O’Leary, 1993). A detailed discussion of isotope effects can be found in Farquhar et al. (1989a).

C. Carbon Isotope Discrimination

Isotope composition is not always a convenient way for expressing results. Indeed, Farquhar and Richards (1984) proposed for convenience the use of carbon isotope discrimination (Δ) defined as the deviation of isotope effects α from unity, because the isotope effect usually exceeds unity by a small number. Hence, it is more convenient to express the results as

\[ \Delta = \alpha - 1. \]  

In the case of plant processes and photosynthesis the source is represented by air CO2 with isotope abundance ratio Ra, while the product is plant material or photosynthetic products with isotope ratio Rp. Hence, from Eq. (3)

\[ \Delta = R_a/R_p - 1. \]  

The value of Δ is calculated from the measured carbon isotope compositions of air CO2 (δa) and of plant material (δp). Therefore, using Eq. (5) and the definition of Eq. (2) for air CO2 \[ \delta_a = R_a/R_p - 1 \] and for plant material (δp = Rp/Ra - 1), carbon isotope discrimination is given by

\[ \Delta = \frac{\delta_a - \delta_p}{1 + \delta_p} \]  

In plants, carbon isotope composition is a negative value, while discrimination is generally positive. That is, plants generally discriminate against 13C. The value of Δ is independent of the isotope composition of the standard used in measurements of isotope composition. Furthermore, Δ has also the advantage of being independent of the source air CO2 composition, and it is a measure of the intrinsic isotopic fractionation by plants. This is especially relevant for comparing experiments where the isotope compositions of the air CO2 differ, such as with free atmospheric CO2 and, by contrast, industrial CO2 produced from fuel combustion. It has been pointed out (O’Leary, 1981) that the simultaneous use of isotope composition and discrimination may be confusing because of the opposite sign. Hence, as previously suggested (Farquhar et al., 1989a), it is always preferable that results are expressed as discrimination Δ. In cases where the atmospheric CO2 composition is not known, the use of δp is acceptable. However, if the uncertainty in δa is small, it may be less confusing to state an assumed value for δa and express the results in terms of Δ.

It is noteworthy that both δ and Δ are dimensionless. For both isotopic composition and discrimination, results are expressed for numerical convenience as the value times 10^-3 or ‰ (per mil). For example a Δ value of 0.025 is usually presented as 25 × 10^-3 or 25‰. However, these notations are not strictly units as often mistakenly stated in the literature. Nevertheless, in certain applications involving the global carbon cycle, for example, it is convenient to work with the product of a flux and either an abundance or a discrimination, for which the notation, say, of Gt ‰ a^-1 is convenient.

D. Instantaneous ‘On Line’ Measurements of Δ During Photosynthesis

Since the photosynthetic process generally discriminates against 13CO2, when the source CO2 for a plant or a leaf is not an unlimited reservoir, there also will be an effect of this fractionation process on the CO2 in the air. Therefore, the CO2 remaining after photosynthetic CO2 uptake by the leaf will be enriched in the heavier isotope. Hence, if the isotope composition of the air is measured before and after this enrichment has occurred, it is possible to calculate the discrimination during photosynthesis.

This principle can be used either in a closed or in an open system. In the former method, a plant or a leaf is enclosed in a closed container with a given amount of CO2 and known δa. The plant is allowed to take up CO2 for a short period and then the new isotope composition of the remaining CO2 is measured. The earliest application of this method was reported by O’Leary et al. (1986), and the fractionation during photosynthesis was calculated solving a series of equations. The open system has been more extensively applied, using open gas exchange systems and well-stirred leaf chambers, as it overcomes the problem of continuous changes in
CO₂ concentration and humidity. In this case, the isotope composition of the air is measured before (δ₀) and after (δₑ) it passes over the leaf. From the measured concentrations of CO₂ entering (cₑ) and leaving (c₀) the leaf chamber, it is therefore possible to calculate ‘on-line’ Δ as (Evans et al., 1986)

\[
\Delta = \frac{\xi(\delta_e - \delta_e)}{1 + \xi(\delta_o - \delta_e)},
\]

where \(\xi = c_e/(c_e - c_o)\). This method has been extensively used and tested in a large number of species with different photosynthetic pathways. An expression for on-line Δ accounting for CO₂ respired and photorespired and subsequently refixed has been used by Gillon and Griffiths (1997).

Measurement of Δ on-line gives an instantaneous estimate of all the fractionation processes associated with net CO₂ uptake and has the advantage of being non-destructive. Hence, discrimination in an individual leaf can be measured several times and in different conditions to estimate the effects of environmental factors.

E. Carbon isotope Composition of Source CO₂

The carbon source for the terrestrial photosynthetic process is the CO₂ of the surrounding air. This CO₂ after several physical and chemical processes is carboxylated into photosynthetic products. In the case of field grown plants, the source CO₂ is represented by atmospheric CO₂ after possible changes due to variability in turbulent mixing within the canopy boundary layer. The isotope composition of atmospheric CO₂ at present is close to −8‰, with slight variations depending on the measuring stations and the time of the year (Francey et al., 1995; Keeling et al., 1995). Carbon isotope composition of atmospheric CO₂ is closely correlated with the CO₂ concentration of the atmosphere (Fig. 1), although climatic events such as El Niño/Southern Oscillation may induce changes in CO₂ concentration not reflected in the isotope record (Francey et al., 1995). Carbon isotope composition of atmospheric CO₂ is closely correlated with the CO₂ concentration of the atmosphere (Fig. 1), although climatic events such as El Niño/Southern Oscillation may induce changes in CO₂ concentration not reflected in the isotope record (Francey et al., 1995).

As already pointed out above, atmospheric CO₂ concentration and isotope composition are subjected to variation, both seasonally and with latitude. Seasonal variation is mostly attributable to variation in the balance between photosynthesis and respiration. The amplitude of such variation in δᵣ can be as wide as 1‰, and is dependent on latitude, being greatest at northern latitude. There is a decreasing trend of fluctuation in δᵣ going from the measurement station at Point Barrow (71°N), to those at Mauna Loa (21°N), Samoa (14°S), Cape Grim (41°S), and South Pole (90°S), in that order (Francey et al., 1995). There is also a gradient in the average δᵣ with latitude, with most negative values occurring at northern latitudes, where the majority of combustion of fossil fuel occurs.

There is also a long-term decline in δᵣ associated with the increasing CO₂ concentration of the atmosphere due to fossil fuel burning and deforestation. This has been demonstrated (Fig. 1) from long term measurements of concentration and isotope composition (Keeling et al., 1979; Roeloffzen et al., 1991) and from ice core records (Friedli et al., 1995). The isotope composition of the atmosphere was about −6.4‰ before the industrial revolution and has gradually become more depleted in ¹³C because of human activities. Reconstruction of past isotope composition of the atmosphere has also been performed using the isotope composition of C₃ plants (Marino and McElroy, 1991; Marino et al., 1992), based on the assumption that discrimination in these plants is relatively constant across a range of environmental conditions. As discussed later, this assumption is not always valid.

Because of spatial and temporal variations in the
isotopic composition of atmospheric CO₂, it is sometimes important to measure the actual δ₁₈O in each experimental condition. This is especially relevant in experiments where plants are grown in artificial atmospheres, such as in growth chambers, open top chambers and experiments under free atmosphere CO₂ enrichment (FACE). In these conditions, the isotope composition is usually dependent on the relative mixing proportion of atmospheric CO₂ and tank CO₂, which can be derived from different sources such as fossil fuel combustion or gas from natural CO₂ springs having quite different isotopic composition (Panichi and Tongiorgi, 1975; Gleason and Kyser, 1984). Estimates of exact source isotope composition are also important in the study of long-lived organisms, herbarium specimens and tree rings (Tans and Mook, 1980; Peñuelas and Azcón-Bieto, 1992; Maguas and Brugnoli, 1996).

Relevant variations in δ₁₈O can also occur in the proximity of vegetation. Within or above plant canopies the isotopic composition can change substantially, particularly when turbulent transfer is poor, as a consequence of photosynthetic activity and plant and soil respiration. Photosynthetic fractionation tends to increase the content in of this application should be used with extreme caution (Panichi and Tongiorgi, 1975; Gleason and Kyser, 1984). Estimates of exact source isotope composition are also important in the study of long-lived organisms, herbarium specimens and tree rings (Tans and Mook, 1980; Peñuelas and Azcón-Bieto, 1992; Maguas and Brugnoli, 1996).

The value of δ₁₈O can be measured directly from CO₂ cryogenically purified and injected into the mass spectrometer. As an alternative, it has been proposed (Beerling and Woodward, 1995, Picon et al., 1997) to assess δ₁₈O from the isotope composition of C₄ plants growing in the same environment, based on the method of Marino and McElroy (1991) However, this application should be used with extreme caution since C₄ plants can show large variation in Δ in response to changes in environmental conditions (Henderson et al., 1992; Buchmann et al., 1996).

Isotope composition of source CO₂ can be also changed artificially for conducting labeling studies of plant metabolism (Osmond et al., 1988, Sharkey et al., 1991; Deléens et al., 1994; Loreto et al., 1996). While the use of pure isotopes is restricted to short-term studies because of the high cost, it is relatively easy to find commercial CO₂ tanks with widely different δ₁₈O. Therefore, switching from a certain composition to another can allow the labeling patterns to be followed in different metabolites (Schneider, 1992).

IV. Photosynthetic Fractionation of Carbon Isotopes

Plants are generally depleted in ¹³C compared to the source atmospheric CO₂, with limited exceptions. Fractionation occurs during diffusion, with ¹³CO₂ diffusing slower than ¹²CO₂ in air, the binary diffusivities being inversely proportional to the square root of the reduced masses of CO₂ and air (Mason and Marrero, 1970). In addition, there are substantial fractionations associated with enzymatic reactions catalyzing carboxylation and decarboxylation. The kinetic isotope effects associated with carboxylations show a wide range, depending on the enzyme involved and thus on the photosynthetic pathway. Indeed, the largest variation in δ₁₈O occurs among plants with different photosynthetic pathways, with differences in Δ around 14–15% between C₄ and C₃ plants. This large difference is due to the fact that ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and phosphoenolpyruvate (PEP) carboxylase, the primary carboxylating enzymes in C₃ and C₄ plants, respectively, show different intrinsic kinetic isotope effects. Furthermore, while the substrate for Rubisco is gaseous CO₂, that for PEP carboxylase is HCO₃⁻, with a significantly different isotope composition because of isotope effects during hydration of CO₂ to HCO₃⁻. Plants exhibiting CAM metabolism show largely variable isotope composition, depending on the proportion of total carbon fixed by conventional C₄ photosynthesis during the day.

Variations are more pronounced among C₃ species, than among C₄ species. The difference in behavior among plants with different CO₂ fixation pathways is specifically linked to the kinetic properties of the primary carboxylating enzymes involved and to consequent differences in the dependency of Δ on diffusive components, while variation within species is attributable to genetic and environmental effects. Hence, within a certain range of the ratio of intercellular and atmospheric partial pressures of CO₂, C₃ species show higher variability in Δ than C₄ species.
A. Discrimination During C₃ Photosynthesis

Carbon isotope discrimination in C₃ species is determined by several fractionation processes occurring during diffusion of gaseous CO₂ from the free atmosphere, through boundary layers (canopy and leaf boundary layers) and through stomata, to the intercellular air spaces. Subsequently, fractionation occurs during dissolution and liquid phase diffusion to the sites of carboxylation, and during carboxylation itself. Various kinetic and equilibrium isotope effects are involved in these steps. Several authors have developed mathematical models describing the fractionation associated with C₃ photosynthesis (Vogel, 1980; O’Leary, 1981; Farquhar et al., 1982a). These models are similar in concept and structure and divide the fractionation associated into diffusive and carboxylation components. The diffusion processes are always reversible to some extent, while the carboxylation step is irreversible. Hence, carbon isotope discrimination can take place only before the carboxylation reaction occurs. Carbon isotope discrimination is conveniently described by the model of Farquhar et al. (1982a), which has been extensively tested in various species and experimental conditions. Carbon isotope discrimination is given by

\[
\Delta = a_b \frac{p_a - p_i}{p_a} + a \frac{p_s - p_i}{p_a} + (e_s + a_1) \frac{p_i - p_c}{p_a} + \frac{e R_d}{b} \frac{p_e - k f \Gamma^*}{p_a}
\]

(8)

where \(p_a\), \(p_o\), \(p_i\) and \(p_c\) are the CO₂ partial pressures in the free atmosphere, at the leaf surface, in the intercellular air spaces before it enters in solution, and at the sites of carboxylation, in that order; \(a_b\) is the discrimination occurring during diffusion in the boundary layer (2.9%, Farquhar, 1980); \(a\) is the fractionation occurring during diffusion in air (4.4%, Craig, 1954); \(e_s\) is the fractionation occurring when CO₂ is dissolved in solution (1.1%, at 25 °C, Vogel, 1980); \(a_i\) is the fractionation occurring during diffusion in the liquid phase (0.7%, O’Leary, 1984); \(b\) is the net discrimination occurring during carboxylations in C₃ plants; \(e\) and \(f\) are the fractionations possibly occurring during dark respiration (\(R_d\)) and photorespiration, respectively; \(k\) is the carboxylation efficiency and \(\Gamma^*\) is the CO₂ compensation point in the absence of dark respiration (Brooks and Farquhar, 1985).

The value of \(b\) in Eq. (8) is not simply that of discrimination associated with carboxylation by Rubisco, because even in C₃ plants a variable proportion of the carbon is fixed by PEP carboxylase (Nalborczyk, 1978; Farquhar and Richards, 1984) and by carboxylases other than Rubisco (Raven and Farquhar, 1990). These carboxylases would operate in parallel with Rubisco affecting the isotopic composition of the overall C fixed. Anaplerotic CO₂ fixation by PEP carboxylase is the most significant contribution other than Rubisco, to the total carbon budget in C₃ species, but other carboxylases may also account for small amounts of carbon fixed. Raven and Farquhar (1990) indicated that, in addition to Rubisco and PEP carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC), acetyl CoA carboxylase and carbamyl phosphate synthetase (CPS) may also account each for at least 1% of total carbon acquisition in at least some O₂-evolving eukaryotes. Hence, taking into account that \(\beta\) is the relative proportion of carbon fixed by PEP carboxylase, \(\gamma\) is that fixed by CPS and \(\epsilon\) is that fixed by Acetyl CoA carboxylase, with associated fractionation factors \(b_\beta\) for Rubisco, \(b_\gamma\) for PEP carboxylase, \(b_\gamma\) for CPS and \(b_\epsilon\) for Acetyl CoA carboxylase, then the discrimination \(b\) associated with all carboxylation reactions is given by

\[
b = b_\beta (1 - \beta - \gamma - \epsilon) + b_\gamma \beta + b_\gamma \gamma + b_\epsilon \epsilon.
\]

(9)

This equation would allow the study of the effects of different carboxylases on \(\Delta\) of plants, taking into account the extent of organic acid synthesis and the influence of different nitrogen source for growth (Raven and Farquhar, 1990). Nevertheless, while Eq. (9) can be used for theoretical considerations about the true value of \(b\), determination of the extent of organic acid synthesis as proposed by Raven and Farquhar (1990) would only be valid provided that subsequent decarboxylations have no effect on \(\Delta\) of such products. However, decarboxylation reactions may have large effects on organic acid \(\Delta\) (O’Leary et al., 1992, see below), particularly if the C atom added, for example, by PEP carboxylase is subsequently decarboxylated. In such a case, the resulting carbon skeleton would purely show a C₃ type isotopic signature. However, based on the above assumptions, Raven and Farquhar (1990) calculated a decrease in \(\Delta\) due to these enzyme activities ranging...
between 0.24% for NH₄⁺ assimilation, and 2.80% for NO₃⁻ assimilation and reduction in the shoot with organic acid salt synthesis for acid-base balance and for further free organic acid requirements. Recent studies have shown that variation in isotope composition related to the source of N nutrition are not always in the predicted direction (Martinez-Carrasco et al. 1998, Yin and Raven, 1998). In addition, nitrogen source also induces changes in p/K with evident complication in the interpretation of results.

Among non-Rubisco carboxylases, the contribution by PEP carboxylase is probably predominant in higher plants. Therefore, if the contribution of carboxylases other than Rubisco and PEP carboxylase is ignored, then Equation (9) can be written in a simpler form as

\[ b = b_3 (1 - \beta) + b_4 \beta = b_3 - (b_3 - b_4) \beta, \]

which is the net discrimination during carboxylations in C₃ plants as proposed by Farquhar and Richards (1984). The isotope effect associated with carboxylation by Rubisco was measured by Roeske and O’Leary (1984). They reported a value of \( \alpha = 1.029 \pm 0.001 \) with respect to dissolved CO₂. If this value is corrected for the isotope effect during dissolution of CO₂, then \( b_3 \) is approximately 30% compared to gaseous CO₂ (Brugnoli et al. 1988; Farquhar et al. 1989b). The isotope effect associated with Rubisco carboxylation was also found to be pH dependent (Roeske and O’Leary, 1984), being 1.026 at pH 9.0 and 1.030 at pH 7.0. However, it is unlikely that variations in stromal pH induce significant variation in discrimination by the enzyme. Guy et al. (1993) found a value of \( b_3 = 30.3\% \) for spinach enzyme, while the values for *Rhodospirillum rubrum* and *Anacystis nidulans* enzymes ranged between 19.6 and 23.0%, depending on reaction conditions, and especially on Mg²⁺ concentration. A lower isotope effect for Rubisco from *R. rubrum* was also reported by Roeske and O’Leary (1985). It is probable that the lower values are associated with form II of Rubisco, found in some cyanobacteria (Robinson and Cavanaugh, 1995).

The value of \( b_4 \) is a function of temperature, because of the temperature dependence of the equilibrium isotope effect between gaseous CO₂ and HCO₃⁻. At 25 °C \( b_4 = -5.7\% \) (Farquhar, 1983). Hence, the value of \( b_4 \) is dependent on the amount of \( \beta \)-carboxylations and on the difference \( b_3 - b_4 \), which is approximately 36.0%. The value of \( \beta \) may be variable in C₃ plants depending on intrinsic (Nalborczyk, 1978) and environmental factors such as the N source for nutrition (Raven and Farquhar, 1990). An upper limit of \( \beta = 0.1 \) has been proposed by the latter authors, while an average value of about \( \beta = 0.05 \) or lower appears realistic for most C₃ plants (Farquhar et al., 1989b). Using the latter value, Eq. (10) would give a value of \( b = 28.2\% \), which is 1.8% lower than the discrimination by Rubisco only. Nevertheless, as discussed above, if a proportion of carbon fixed by PEP carboxylase is subsequently decarboxylated, the true value of \( b \) would fall between 28.2% and 30%. Further studies are needed on this subject.

Other sources of uncertainty in the determination of \( \Delta \) are represented by the fractionation processes associated with respiration and photosynthesis, the terms e and f of Eq. (8). Direct measurements of e and f are difficult, particularly in the light during photosynthesis. Indirect estimates using on-line \( \Delta \) indicated that e is not significantly different from zero (von Caemmerer and Evans, 1991). Recently, the possible fractionation associated with dark respiration was measured on mesophyll protoplasts of bean and corn, using different culture solutions containing glucose, fructose and sucrose with known δ¹³C (Lin and Ehleringer, 1997). From the comparison of δ¹³C of the substrate and respired CO₂ it was shown that, at least in isolated protoplasts, there is no significant fractionation during mitochondrial dark respiration. Consistent results have also been reported by Gillon and Griffiths (1997). However, recent experiments on intact bean leaves have shown a significant enrichment in δ¹³C by about 6% in the CO₂ respired in the dark compared to leaf sucrose, indicating a substantial fractionation associated with respiration (Duranceau et al., 1999).

The value of f was estimated to be 7%, measuring the air isotope composition at compensation point (Rooney, 1988). von Caemmerer and Evans (1991) also suggested that f may be non-zero and pointed out that the intrinsic problem in on-line \( \Delta \)estimates is that a value of f = 5% would lead to a measurable effect of only 0.8%. Recently, Gillon and Griffiths (1997) have used on-line measurements manipulating δ³C either during growth or during \( \Delta \)measurements to obtain differences in δ between CO₂ respired and photosynthesized and that of ambient air. They found f to be variable among species, 3.3% in wheat and 0.5% in bean. However, subsequent measurements by the same authors (J. Gillon and H. Griffiths,
personal communication) gave values nearer to that estimated by Rooney (1988) and more constant across different species. Nevertheless, the fractionation during CO₂ release by plants is not yet well characterized and more quantitative studies are needed.

The formulation of Eq. (8) takes into account, step by step, the different fractionation factors associated with diffusion of CO₂ in the gas phase from still air to the intercellular air spaces, then the isotope effects associated with dissolution and liquid phase diffusion and with carboxylations and those occurring during photorespiration and dark respiration. However, in many studies a simplified version of Eq. (8) is used taking into account that the effects on discrimination associated with CO₂ transfer from the intercellular air spaces to the sites of carboxylation and those associated with photorespiration and dark respiration are often small and can be neglected (Farquhar et al., 1989a; O’Leary, 1993). Then the discrimination in C₃ plants is given by

\[ \Delta = a + (b - a) \frac{p_l}{p_a}. \]  

(11)

This simplification may also work roughly when the drop in pCO₂ from the intercellular spaces to the sites of carboxylation is not small, provided the source of variation in Δ is stomatal. Eq. (11) is a useful approximation and predicts a linear relationship between Δ and p_l/p_a (Fig. 2). It shows that when p_l/p_a is small, Δ is dominated by diffusional fractionation (i.e. 4.4%), while when p_l/p_a is large then Δ is dominated by fractionation due to carboxylation (i.e. about 28%). It is noteworthy that p_l/p_a is determined by the balance between photosynthetic capacity and stomatal conductance, i.e., the balance between the demand and the supply functions for photosynthesis (Farquhar and Sharkey, 1982). Therefore, a low value of p_l/p_a can be either determined by low conductance or by relatively high biochemical photosynthetic capacity, or both.

B. Discrimination During C₄ Photosynthesis

Carbon isotope discrimination during photosynthesis in C₄ plants is more complex, since PEP carboxylase is the primary carboxylating enzyme, and CO₂, after conversion to HCO₃⁻, is fixed through this pathway in mesophyll cells. After the transfer of C₄ compounds to the bundle sheath cells and subsequent decarboxylation, CO₂ enters the C₃ pathway via Rubisco carboxylation. If the bundle sheath cells were absolutely gas tight, all the CO₂ produced from decarboxylation would be subsequently refixed by Rubisco and there would be no opportunity for discrimination in the carboxylation of RuBP. In fact, the system is not perfected since some CO₂ leaks out from bundle sheath and, as a consequence, Rubisco can discriminate against ¹³C. Several models have been developed to describe discrimination during C₄ photosynthesis (Peisker, 1982; Deleens et al., 1983; Farquhar, 1983, Peisker and Henderson, 1992). As in C₃ plants, fractionation also occurs in C₄ leaves as CO₂ diffuses from the atmosphere to the sites of primary carboxylation. After gas phase diffusion, CO₂ enters in solution and is converted to HCO₃⁻, which is the substrate for PEP carboxylase. As stated above, the associated fractionation processes are temperature dependent. At equilibrium, during dissolution of CO₂ into water at 25 °C, ¹³CO₂ concentrates in the gas phase by 1.1% (Mook et al., 1974, O’Leary, 1984). On the other hand, at equilibrium ¹³C concentrates in HCO₃⁻ by 9.0% (Mook et al. 1974). Hence the net equilibrium
fractionation during dissolution and hydration of CO$_2$ at 25°C is $-7.9\%$, while $-8.5\%$ at 20°C and $-7.4\%$ at 30°C (Farquhar, 1983). Discrimination by PEP carboxylase is 2.2% against H$^{13}$CO$_3^-$, and therefore the net fractionation with respect to gaseous CO$_2$ is $\delta_g = -5.7\%$ (at 25°C), which actually favors $^{13}$C. A simple expression for calculating $\delta_g$ as a function of temperature was presented by Henderson et al. (1992). Discrimination by PEP carboxylase is nearly independent of the phosphorylation state and of pH (Chollet et al., 1996). A further possibility for discrimination is that associated with Rubisco carboxylation ($\delta_b$) in bundle sheath cells, because a proportion $\phi$ of CO$_2$ released by decarboxylation can actually leak out of the bundle sheath. Finally, discrimination can occur during leakage of CO$_2$ from bundle sheath. By analogy with the model for C$_3$ photosynthesis (Eq. (10)), discrimination in C$_4$ plants (Farquhar, 1983; Henderson et al., 1992) can be written as

$$\Delta = a \frac{p_a - p_s}{p_a} + a \frac{p_s - p_i}{p_a} + (e_3 + a_1) \frac{p_i - p_m}{p_a} + (b_4 + b_3 - s \phi) \frac{p_m}{p_a},$$

(12)

where $p_a$, $p_s$, $p_i$, and $p_m$ are the CO$_2$ partial pressures in air, in the boundary layer, in the intercellular air spaces, and in the mesophyll cytoplasm (assumed here to be in equilibrium with bicarbonate), in that order; $b_4$ and $b_3$ are the fractionations associated with PEP and RuBP carboxylations, respectively, and $s$ is the fractionation during leakage. Assessment of an appropriate value for $s$ is complicated (Farquhar 1983), but Henderson et al. (1992) took the value to be close to the sum $(e_3 + a_1)$ (i.e., $=1.8\%$).

Another model for discrimination during C$_4$ photosynthesis proposed by Peisker and Henderson (1992), although considering four types of limitations to the overall photosynthesis rate, is conceptually similar to that of Eq. (12). Carboxylations by Rubisco and PEP carboxylase were considered to be limited either by maximum enzyme activities or by maximum substrate regeneration capacities, but these limitations are still described by Eq. (12). The authors used this model to try to interpret the effects of environmental and genetic determinants on discrimination during C$_4$ photosynthesis.

A simplified version of Eq. (12) ignores the isotope effects due to CO$_2$ transfer in the boundary layer and during transfer from the intercellular air spaces to the mesophyll cells, since these effects were thought to be relatively small (Farquhar, 1983; Henderson et al., 1992). Then the discrimination occurring in C$_4$ photosynthesis is given by

$$\Delta = a + (b + \phi(b_3 - s) - a) \frac{p_i}{p_a}.$$

(13)

The slope of the relationship between $\Delta$ and $p_i/p_a$ (Fig. 2) is therefore dependent on the relative amount of leakage from bundle sheath cells. Henderson et al. (1992) estimated $\phi$ in a range of species using this relationship with instantaneous on-line measurements of $\Delta$. They found values of $\phi$ of about 0.2 for several species, including NAD-ME (malic enzyme) dicots, NADP-ME dicots, and monocots either of the NADP-ME and NAD/PCK (phosphoenolpyruvate carboxy-kinase) types. The species analyzed by these authors also included two species possessing and four species lacking a suberized lamella in the bundle sheath cells. On the other hand, they found significantly higher values of $\phi$ ranging from 0.25 to 0.30 for the PCK monocot Chloris gayana with suberized lamella, and the NAD-ME monocot Eleusine coracana, lacking the suberization of bundle sheath. Hence, it was concluded that leakiness cannot unequivocally be associated with different decarboxylation pathways or with the presence of a suberized lamella in the bundle sheath, probably because leakiness is determined not only by the physical conductance of bundle sheath to CO$_2$, but also by the relative activities of PEP carboxylase and Rubisco. This report contrasts partially with previous views indicating a causal relationship between the proportion of leakiness, or the quantum yield of CO$_2$ assimilation, and the presence of a suberized lamella (Ehleringer and Pearcy, 1983; Ohsugi et al., 1988; Farquhar et al., 1989a). Variation in $\phi$ had been also attributed to different decarboxylation pathways, with NAD-ME showing greater leakage than NADP-ME and PCK type C$_4$ grasses (Farquhar et al., 1989a). Such variation in $\phi$ among different decarboxylation pathways was not observed in the work by Henderson et al. (1992). These authors also found that the value of $\phi$ estimated from on-line $\Delta$ measurements differed significantly from that estimated from dry matter $\Delta$, which gave much higher $\phi$ values ranging between 0.31 to 0.47. The latter range is similar to that found by previous authors (Hattersley, 1982; Farquhar, 1983). The difference between the two estimates was attributed to fractionation occurring after photosynthesis, and possibly during night respiration (Lin and Ehleringer,
observed in sugarcane genotypes (Meinzer et al., 1994). These changes in ϕ were attributed to decrease in Rubisco activity relative to the C₄ cycle capacity, rather than to changes in physical characteristics of bundle sheath cells. Similar results were obtained on several sugarcane clones exposed to different irrigation, with drought induced decline in the ratio of Rubisco to PEP carboxylase activity explaining most of the decrease in \( \Delta \) and ϕ (Saliendra et al., 1996). Genetic variation in \( \Delta \) attributable either to variation in ϕ or in \( p/p_s \) or both, was also observed in Sorghum bicolor (Hubick et al., 1990). On the contrary, increasing salinity was found to induce a decline in \( \Delta \) and in the proportion of leakiness in several Atriplex species growing along a gradient of soil salinity (Walker and Sinclair, 1992).

In a recent study (Buchmann et al., 1996), large variations in \( \Delta \) induced by different light and water availability were reported for several C₄ grasses with different decarboxylation pathways. Discrimination was found to increase with decreasing light at PFD lower than 700 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and with drought stress. These changes in \( \Delta \) were explained by dramatic changes in the estimated ϕ, ranging between 0.22 to 0.75. The highest proportion of leakage was calculated for grasses of NAD-ME type, followed by PCK grasses, while those of the NADP-ME showed the lowest ϕ values. Nevertheless, it is possible that the observed variation in \( \Delta \) was not entirely attributable to increased ϕ but also to changes in \( p/p_s \).

Increased \( \Delta \) and ϕ and reduced photosynthetic efficiency and quantum yield induced by nitrogen stress in sugarcane were accompanied by decreased ratio of Rubisco to PEP carboxylase activities, leading to an imbalance between C₃ and C₄ cycles (Ranjith et al., 1995; Meinzer and Zhu, 1998). In contrast with this observation, decreased \( \Delta \), suggesting also a decreased ϕ under low N nutrition, was observed in Echinochloa frumentacea (Wong and Osmond, 1991), though \( p/p_s \) was not measured in this study. Indeed, to estimate changes in ϕ it is absolutely necessary to measure possible variation in \( p/p_s \) in order to avoid misleading interpretations.

Most of the reported variations in \( \Delta \) and in ϕ in C₄ plants were explained by variations in Rubisco activity leading to a decrease in the ratio of Rubisco to PEP carboxylase activities. Recently, von Caemmerer et al. (1997) have shown that the reduction of Rubisco content in transgenic Flaveria bidentis, with an antisense gene directed against the Rubisco mRNA, caused an increase in \( \Delta \) mostly mediated by an
increase of bundle sheath CO₂ partial pressure and consequent increase in \( \phi \). Although the \( p_t/p_a \) ratio also increased, \( \phi \) for transgenic plants was 0.37, in comparison with 0.24 in control plants. This increase in \( \phi \) was evident from on-line \( \Delta \) measurements and from the isotope composition measured directly on bulk leaf material. An imbalance between \( C_3 \) and \( C_4 \) cycles may be responsible of the increased \( \phi \) through, a down regulation of the \( C_4 \) cycle may also have occurred through a reduction in PEP regeneration rate (von Caemmerer et al., 1997).

In other studies, increasing \( \Delta \) values from the base to the tip of corn leaves were attributed to an accompanying increase in PEP carboxylase content (Sasakawa et al., 1989), although an increase in Rubisco content from the base to the tip was also found. In sugarcane (Meinzer and Saliendra, 1997) a similar pattern of longitudinal variation in leaf \( \Delta \) was entirely attributed to variation in \( p_t/p_a \) with \( \phi \) being constant over the leaf. Hence, coordinated variations in Rubisco and PEP carboxylase activities, were correlated with the prevailing light intensity, and did not affect the leakage from bundle sheath cells. Hence, it is clear that, in addition to anatomical characteristics, the ratio of Rubisco to PEP carboxylase activities controls carbon isotope discrimination and leakiness in \( C_4 \) plants. Therefore, variation in the amounts and activities of the enzymes, if coordinated, will not affect \( \phi \).

C. Discrimination in CAM Plants

Carbon isotope discrimination in plants possessing Crassulacean acid metabolism has been reviewed previously (O’Leary, 1988; Farquhar et al., 1989a; Griffiths, 1992). Modeling carbon isotope discrimination in CAM plants is complicated by the presence of \( C_3 \) and \( C_4 \) cycles, temporally separated. Furthermore, PEP and RuBP carboxylations may sometimes partially coexist during short periods of the day. Diffusion limitation imposed by partial stomatal closure can have a substantially different impact on fractionation depending when in the CAM cycle it occurs. It may lead to an increased \( \Delta \) during nocturnal CO₂ fixation, and to a smaller \( \Delta \) during the \( C_3 \) phase, according to the models presented above. During dark CO₂ fixation by PEP carboxylase (phase I, Osmond, 1978), with storage of malate in the vacuole, carbon isotope discrimination would be essentially the same as for \( C_4 \) plants, with no leakage (Farquhar, 1983). However, at the end of the dark period, and beginning of the daytime, there is a shift in carboxylation from PEP carboxylase to Rubisco (phase II). The former enzyme is gradually deactivated by malate efflux from the vacuole and by light, preventing futile cycling of decarboxylation products (Osmond et al., 1988), and the increased CO₂ concentration leads to stomatal closure by the end of phase II. During this phase, discrimination is expected to show values intermediate between those typical for \( C_3 \) and \( C_4 \) photosynthesis. Subsequently, CO₂ originating from decarboxylation of malic acid is fixed by Rubisco (Phase III), with a process analogous to that occurring in the bundle sheath in \( C_4 \) species. Hence, if no efflux of CO₂ occurred with tightly closed stomata, there would be no opportunity for expressing the fractionation by Rubisco. However, there is always some leakage of CO₂ because stomata are not completely closed, and therefore discrimination by Rubisco occurs. Because of this discrimination, the CO₂ lost is expected to be very heavy (O’Leary, 1988). The discrimination in such cases would be \( \phi (b_3 – s) \) leading to an enrichment in the CO₂ leaking out by \( (b_3 – s) \). It is noteworthy that \( p_t \) can be as high as 2–4 kPa (Osmond, 1978, Griffiths, 1992).

In the late afternoon, toward the end of the light period, when all of the malic acid associated with the CAM cycle has been decarboxylated, stomata may re-open. Then CO₂ fixation may occur directly through Rubisco (phase IV, Osmond, 1978). This \( C_4 \) activity can contribute substantially to the total carbon gain, especially under optimal conditions. A further complication in quantitative approaches to discrimination in CAM plants is represented by the contribution of respiratory CO₂ refixed, particularly during phase I (Griffiths, 1992). It has been reported that in Kalanchoe tubiflora, at least 15% of the total malate was formed from respiratory CO₂ at 15 °C, while this contribution increased to 49% at 25 °C (Kalt et al., 1990). Furthermore, randomization of the isotope signature in malate by fumarase can have significant and variable effects on the isotope ratio of CO₂ decarboxylated (O’Leary, 1988; Osmond et al., 1988; Kalt et al., 1990). Measurements of instantaneous \( \Delta \) during the various phases of CAM activity in Tillandsia usrictulata (Griffiths et al., 1990) showed values of \( \Delta \) ranging between 4.4 and 6.6‰ during phase I. More recently, on-line \( \Delta \) measurements during phase I in Clusia minor, a \( C_3 \)-CAM intermediate, indicated values of \( \Delta \) ranging from −6‰ with wide open stomata, to 4.4‰ with closed
stomata (Roberts et al., 1997). These values are close to the theoretical limits of $\Delta$ set for $p_i/p_a$ values of 1 and 0, respectively. Subsequently, as expected, a shift in $\Delta$ toward $C_4$ values has been reported for phase II (Griffiths et al., 1990). During this phase $\Delta$ was initially around 10%, indicating an immediate contribution of Rubisco, which become proportionally more pronounced as $CO_2$ fixation declined, as indicated by a $\Delta$ of about 21% at the end of phase II. A surprisingly high discrimination was indeed found in this study during phase III, with a $\Delta$ of about 64%. This would indicate that the $CO_2$ leaking out of the leaf is highly enriched in $^{13}C$ ($\delta$ of about +52%). However, this $\Delta$ value is much greater than predicted from current knowledge, with the estimated value of leakage of about 0.012, and it is rather difficult to explain. The intrinsic difficulty and inaccuracy in the on-line $\Delta$ measurements because of low $CO_2$ exchange rate during this phase may partly explain these results. Finally, the same authors reported values characteristic of the $C_3$ pathway during phase IV, because of direct Rubisco contribution to $CO_2$ uptake. However, recent studies have shown that the relative contribution of Rubisco and PEP carboxylase to the isotope signature of carbon during phase II and IV can vary significantly among CAM and $C_3$-CAM intermediates. In the latter a substantial $C_4$ contribution has been reported during these phases, although also matched by an increased $C_4$ capacity compared to constitutive CAM (Borland and Griffiths, 1997).

Hence, carbon isotope discrimination can give information on the $C$ fixation pathway being used, showing the relative importance of $C_4$ photosynthesis and CAM engagement. This is particularly relevant for studying the induction of CAM in $C_3$-CAM intermediates (Borland et al. 1994).

Recent experiments with the obligate CAM Kalanchoe daigremontiana (Maxwell et al., 1997) have shown that the leaf internal conductance to $CO_2$ in CAM can be as low as 0.05 mol m$^{-2}$s$^{-1}$, much lower than the lowest limit found in $C_3$ species (see Chapter 14, Evans and Loreto). This low conductance, measured during $C_3$ fixation in phase IV, was explained as a consequence of the leaf or stem succulence, considered an adaptation for water conservation and a mean to increase the buffering capacity to store $C_4$ acids in vacuoles. Hence there would be a tradeoff for $CO_2$ diffusion and fixation by both PEP carboxylase and Rubisco (phases I, II, IV) and the succulent mesophyll required for $C_4$ acid storage (Maxwell et al. 1997).

**D. Discrimination in $C_3-C_4$ Intermediates**

Physical and biochemical factors underlying carbon isotope discrimination in $C_3-C_4$ intermediates have been recently studied in detail, and models have been developed describing the discrimination during photosynthesis in these plants (Farquhar et al., 1989a; von Caemmerer, 1989). These aspects have been recently covered by an excellent review (von Caemmerer, 1992).

In $C_3-C_4$ intermediates carbon fixation is performed by Rubisco in the mesophyll and in the bundle sheath cells. While the discrimination by Rubisco is expressed in both cell types, the source $CO_2$ may be rather different. The source $CO_2$ in the mesophyll cells is comparable to that of $C_3$ leaves, with effects of diffusion fractionation with respect to $CO_2$ in external air. In the bundle sheath cells, the source $CO_2$ is partly derived from decarboxylation of glycine and $C_4$ compounds, working as a shuttle between mesophyll and bundle sheath cells. In addition, similarly to $C_4$ photosynthesis, the expression of Rubisco fractionation in the bundle sheath is dependent on the amount of $CO_2$ leakage, $\phi$. Hence, $\Delta$ is given by (von Caemmerer, 1992)

$$\Delta = a + \left( b_3 - a \right) \frac{p_i}{p_a} + \frac{A_s}{A} \left[ (b_3 - s)\phi + \left( b_4 - b_3 \right) \frac{V_p}{S} \right] \frac{p_i}{p_a}, \quad (14)$$

where $A$ is the overall net assimilation rate by the leaf, $A_s$ is the assimilation rate in the bundle sheath, $V_p$ is the rate of PEP carboxylations and $S$ is the rate of $CO_2$ supply to the bundle sheath from the mesophyll and is the sum of glycine and $C_4$ acid decarboxylations. This equation shows that $\Delta$ in $C_3-C_4$ intermediate is the sum of $\Delta$ in $C_3$ photosynthesis and a second term which depends on the relative proportion of $CO_2$ fixation in the bundle sheath, on the proportion of leakiness and on the relative contribution of glycine and $C_4$ acid decarboxylation in the bundle sheath.

Experimental results have shown that $\Delta$ in $C_3-C_4$ intermediates is $C_3$ like (Hattersley et al., 1986; von Caemmerer and Hubick, 1989; Araus et al., 1991).
because only a small proportion of total carbon is fixed in the bundle sheath using the $C_3$ pathway (von Caemmerer, 1992). In this case, Eq. (14) is equivalent to that introduced by Farquhar et al. (1989a).

**E. Discrimination in Lichens and Mosses**

Although several authors have studied $\Delta$ in lichens and bryophytes, the knowledge of the mechanism of isotope fractionation in these organisms is still scanty. The range of variation in $\Delta$ in non-vascular plants is quite large. Variation in $\Delta$ in mosses (e.g., Rundel et al., 1979; Teeri, 1981; Proctor et al., 1992) has been attributed partly to the effect of water content. Particularly, variation in surface water films induces changes in the resistance to $CO_2$ uptake (Rice and Giles, 1994; Williams and Flanagan, 1996). Increasing thickness of such water films would therefore increase the diffusional fractionation component leading to lower $\Delta$ (Cowan et al., 1992). Changes in anatomical properties can also affect the isotope composition of mosses. Studies with mosses of the genus *Sphagnum* (Rice and Giles, 1996) showing different anatomical properties, indicated that the variation in $\Delta$ due to surface water films was higher than that attributable to difference in anatomy. A mechanistic model of gas exchange in mosses, with conductance to $CO_2$ being a function of water content, has been developed recently (Williams and Flanagan, 1998).

Large variation in $^{13}C/^{12}C$ ratios is also found in lichens. Such changes in $\Delta$ have been attributed to the type of photobiont partners and to diffusive resistances to $CO_2$ diffusion. Lichens with green algae (phycobiont) can assimilate $CO_2$ in the presence of high air humidity, while those with cyanobacteria require liquid water, and then will have a greater diffusion limitation and lower $\Delta$ (Lange et al., 1988). Recently, it has been shown that $\Delta$ in lichens falls into three categories: lichens with a phycobiont plus a cyanobacterium limited to structures of the thallus called cephalodia, with $\Delta$ around 24–28‰; lichens with a phycobiont alone and lichens with a cyanobiont alone with two distinct ranges of $\Delta$, both around 15‰ (Mágua et al., 1995). Variation in $\Delta$ may be due to lower discrimination by Rubisco in cyanobacteria compared to algae (Guy et al. 1993). It has also been demonstrated that cyanobacterial lichens possess a $CO_2$-concentrating mechanism (CCM) similar to that of free living cyanobacteria, affecting photosynthetic efficiency and carbon isotope discrimination. The existence of a CCM would therefore cause a marked reduction in $\Delta$ of those organisms (Raven et al., 1990; Mágua et al., 1993). The presence of a CCM has been confirmed by gas exchange studies and online $\Delta$ measurements (Palmquist et al., 1994; Mágua et al., 1995; Smith and Griffiths, 1996), and it has been shown to be related to the presence of chloroplast pyrenoids, structures highly enriched in Rubisco, present in some microalgae (Smith and Griffiths, 1996). In addition to the effects of the CCM, thallus structure, morphology and water content have also been shown to play a significant role in determining differences in $CO_2$ diffusion resistance and consequent changes in $\Delta$ in lichens (Mágua et al., 1995; Mágua and Brugnoli, 1996). Interestingly, differences in $CO_2$ diffusion resistance can cause variation in $\Delta$ within the same thallus (Mágua and Brugnoli 1996; Mágua et al. 1997).

Notwithstanding the increasing interest in this subject, the mechanisms determining $\Delta$ in lichens are still debated. More information is needed on the contribution of CCM activity and of gas diffusion limitation on fractionation, since both processes will invariably cause a decrease in the observed $\Delta$.

**F. Discrimination in Aquatic Plants and Algae**

Carbon isotope composition in plants in the aquatic environment is extremely variable, with values of $\delta$ between −10 and −50‰. These large variations reflect changes in the carbon source for photosynthesis, plant physiological and ecological features, as well as environmental changes such as temperature, pH, salinity and substrate concentration. Despite the large variations in discrimination, most plants of the aquatic habitat possess the $C_3$ photosynthetic pathway, with very limited exceptions (Descolas-Gros and Fontugne, 1990; Farquhar et al., 1989a). Differences in isotope fractionation among these organisms are also caused by the presence or absence of a CCM (Yoshioka, 1997), as discussed for lichens. Carbon isotope discrimination in aquatic organisms has been the object of recent reviews (Descolas-Gros and Fontugne, 1990; Keeley and Sandquist, 1992; Raven, 1992; Fry, 1996) and will be not addressed in further details here.
V. Variation in Isotope Composition Within the Plant

A. Fractionation of Carbon Isotopes in Different Metabolites

Carbon isotope composition can vary substantially among different metabolites and in different organs within the plant. Such differences should be taken into account when analyzing Δ either in the bulk dry matter or in individual metabolites, to study, for example, the diffusional limitation of photosynthesis and variation in the \( \frac{p_0}{p_x} \) ratio. In such cases, variation in Δ due to different organs or chemical composition could be erroneously attributed to variation in \( \frac{p_0}{p_x} \).

While the nature of the differences in isotope composition among some plant metabolites has been elucidated in detail, other differences are not clearly understood.

Differences among plant metabolites are found in all plant species, with different photosynthetic pathways. Figure 3 shows the pattern of variation of Δ in various metabolites in comparison with that in the bulk dry matter, in C₃ species.

One of the most striking differences in isotope composition is found in lipids, which are significantly depleted (5–10%) in \(^{13}\)C compared to bulk plant material. Such differences were first observed in the pioneering work by Park and Epstein (1960). It is now well established that the \(^{13}\)C depletion of fatty acids, and lipids generally, is due to secondary fractionation associated with decarboxylation of pyruvic acid by pyruvate dehydrogenase (DeNiro and Epstein, 1977; O’Leary, 1981; Melzer and Schmidt, 1987). Similarly, fractionation associated with formation of acetyl-CoA by pyruvate dehydrogenase is responsible for the high Δ found in isoprenoids like \( \beta \)-carotene and isoprene (Sharkey et al., 1991). A slight difference in Δ among isoprenoids and fatty acids (Fig. 3) was attributed to the fact that only the carbonyl carbon of acetyl-CoA was depleted in the heavier isotope (Sharkey et al., 1991).

Other relevant differences in Δ among metabolites are found in amino acids, showing an extremely wide range of variation among individual amino acids; Values of Δ ranging between about 6% for aspartic acid and serine to about 23% for leucine were reported by Abelson and Hoering (1961). Different fractionation processes cause such variation. Anaplerotic CO₂ fixation by PEP carboxylase is responsible for the enrichment in \(^{13}\)C of aspartic acid (Melzer and O’Leary, 1987, 1991). This is also the reason for the low Δ of organic acids compared to bulk material in C₃ plants (Raven and Farquhar, 1990; Brugnoli et al., 1997).

In general, secondary plant products show higher Δ than primary products (Schmidt and Gleixner, 1997). It is known that lignin, a secondary product, is strongly depleted in \(^{13}\)C compared to cellulose synthesized from primary photosynthetic products (Galimov, 1985; Gleixner et al., 1993). It has been demonstrated that differences among primary and secondary products can be attributed mainly to the isotope effects associated with fructose-1,6-bisphosphate aldolase reaction (Gleixner et al., 1993; Gleixner and Schmidt, 1997) and possibly to the triose phosphate isomerase reaction (Brugnoli et al., 1988). Differences may also arise due to fractionation during transport of metabolites.

A similar explanation can be found for the difference in Δ between sucrose and starch found in several plant species (Deléens-Provent and Schwebel-Dugué, 1987; Brugnoli et al., 1988; Gleixner et al., 1993).
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1993), with starch being enriched in $^{13}$C compared to sucrose (Fig. 3). There could be an equilibrium isotope effect at triose phosphate, with lighter monomers being preferentially exported from the chloroplast to the cytosol. Hence, sucrose would be more depleted in $^{13}$C than starch accumulated in the chloroplast (Brugnoli et al., 1988). Similarly, there is an equilibrium isotope effect on the aldolase reaction (Gleixner et al., 1993), which may explain the difference between starch and sucrose. In addition, it has been recently demonstrated (Gleixner and Schmidt, 1997) that fractionation during the aldolase reaction is the reason for the non-statistical isotope distribution in the glucose molecule, with a relative enrichment in $^{13}$C in positions C-3 and C-4 compared to C-1 and C-6 positions, previously reported (Ivlev et al., 1987; Rossmann et al., 1991).

Other decarboxylation reactions also involve fractionation processes in secondary metabolism, and are responsible for the observed differences in $\Delta$ in plant metabolites. Such reactions include decarboxylation of glutamate, arginine, acetocacetate, isocitrate, histidine, aspartate and malate with associated fractionation factors quite variable between $-1.1\%$ (isocitrate) and $31\%$ (histidine, O’Leary et al., 1992).

In addition to variation in the isotopic signature of different metabolites, a difference between primary and secondary carbohydrates has been observed. For example it has been found that storage carbohydrate such as sucrose extracted from sugar beet roots (Gleixner et al., 1993) or from rice internodes (A. Scartazza and E. Brugnoli, unpublished) are less discriminated than primary sucrose extracted from leaves. These differences could be attributed to fractionation processes during transport or to fractionation during enzymatic reactions, as discussed above.

The isotope composition of sucrose and starch extracted and purified from leaves should reflect the discrimination of initial $C_3$ products, because the purification removes the products of PEP carboxylase, such as organic acid and amino acids. Hence, the $\Delta$ of sucrose and starch should be higher than the discrimination of total CO$_2$ uptake by an offset of $\beta(b_7-b_4)$, with the same slope of the relationship between $\Delta$ and $p/p_{atm}$ as discussed by Brugnoli et al. (1988, 1997). However, this expected offset was not observed in several experimental situations, indicating indeed that $\Delta$ in sucrose is not significantly different from $\Delta$ measured on-line (Brugnoli et al., 1988, 1997; A. Scartazza and E. Brugnoli, unpublished). These results may be explained by counter-acting effects of other fractionation processes considered in Eq. (10) but not in the simplified version of Eq. (11) (Brugnoli et al., 1988). Another possibility is that the carbon of initial $C_3$ products and that of products of $\beta$-carboxylation are not independent and are rapidly interconverted and randomized during the course of the day. Also subsequent decarboxylation of products of PEP carboxylations may explain these findings. Such effects would cause the $\Delta$ values of the two pool to converge. In the same experiments, $\Delta$ of leaf starch was significantly smaller than that of sucrose and online $\Delta$, probably because of isotope effects at the equilibrium of triose phosphate or during the aldolase reaction, as discussed above. Certainly, further studies are needed to understand the possible source of fractionation in plant metabolites and carbon pools.

Variation in carbon isotope composition among biochemical fractions of CAM or $C_4$-CAM intermediate plants have been studied by Deléens and Garnier-Dardart (1977), O’Leary and Osmond (1980), Borland et al. (1994). These variation is complicated by variable contributions of $C_3$, photosynthesis to individual metabolites, in addition to possible further fractionation processes, occurring after carboxylations. These variations have been reviewed previously (O’Leary, 1988, Griffiths, 1992).

**B. $\Delta$ Variation in Different Anatomical Compartments**

Variation in isotope composition may also occur among different plant organs and compartments. The earliest report of variation among plant parts concerned differences in $\delta$ among leaves and wood of several tree species (Craig, 1953). Subsequently, differences in $\Delta$ among organs were reported for a wide range of species. Table 1 summarizes some examples of studies showing differences in $\Delta$ among different anatomical parts. It is evident that there is a general trend toward an enrichment in $^{13}$C in certain organs such as seeds, in comparison with leaves. Different $\Delta$ values between seeds and leaves were observed in a large range of species, including $C_3$ and $C_4$ species, monocot and dicot crop species. Such differences are rather variable ranging from $0.5\%$ in sunflower to about $5\%$ in wheat. Carbon isotope discrimination in stems is much closer to that in leaves, although in several cases stem $\Delta$ is slightly
Table 1. Carbon isotope discrimination in different plant organs in several plant species. Where available, the mean ± SE or the range of variation is shown. Values of Δ were calculated using the estimated value of δ2 for the appropriate year (Keeling et al., 1979, Friedli et al., 1986).

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
<th>Seeds</th>
<th>Tuber</th>
<th>Wood</th>
<th>Twigs</th>
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<td>Corn</td>
<td>5.2</td>
<td></td>
<td></td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
<td>Gleixner et al., 1993</td>
</tr>
<tr>
<td>Corn</td>
<td>3.8</td>
<td></td>
<td></td>
<td>3.6</td>
<td></td>
<td></td>
<td></td>
<td>Troughton, 1972</td>
</tr>
<tr>
<td>Wheat</td>
<td>18.9 ± 0.6</td>
<td>19.0 ± 0.6</td>
<td>16.4 ± 1.0</td>
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<td></td>
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<td>Farquhar and Richards, 1984</td>
</tr>
<tr>
<td>Wheat</td>
<td>20.5 ± 1.0</td>
<td></td>
<td>15.4 ± 1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Farquhar and Richards, 1984</td>
</tr>
<tr>
<td>Wheat</td>
<td>19.8 - 20.05</td>
<td>19.3 - 20.0</td>
<td>17.9 - 18.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Condon et al., 1992</td>
</tr>
<tr>
<td>Rice</td>
<td>20.1 ± 0.2</td>
<td>19.4 ± 0.1</td>
<td>19.0±0.2</td>
<td>18.7 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td>Scartazza et al., 1998</td>
</tr>
<tr>
<td>Rice</td>
<td>19.6 ± 0.2</td>
<td>18.7 ± 0.2</td>
<td>18.5±0.2</td>
<td>18.3 ± 0.2</td>
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<td></td>
<td></td>
<td>A. Scartazza and E. Brugnoli, unpublished</td>
</tr>
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<td>Barley</td>
<td>17.0 - 21.5</td>
<td>17.2 -21.5</td>
<td></td>
<td>15.0 - 17.2</td>
<td></td>
<td></td>
<td></td>
<td>Acevedo, 1993</td>
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<tr>
<td>Tomato</td>
<td>19.1</td>
<td>18.3</td>
<td>17.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Park and Epstein, 1960</td>
</tr>
<tr>
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<td>18.0</td>
<td>17.7</td>
<td>17.6</td>
<td></td>
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<td>Park and Epstein, 1960</td>
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<td>Sunflower</td>
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<td></td>
<td></td>
<td></td>
<td>E. Brugnoli and M. Lauteri, unpublished</td>
</tr>
<tr>
<td>Bean</td>
<td>20.6 ± 0.1</td>
<td>20.4 ± 0.2</td>
<td>17.6 ± 0.3</td>
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<td></td>
<td></td>
<td>Brugnoli and Lauteri, 1991</td>
</tr>
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<td>21.9</td>
<td>21.1</td>
<td>20.6</td>
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</tr>
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<td>Cowpea</td>
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<td></td>
<td></td>
<td>16.5 ± 0.56</td>
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</tr>
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<td>Gleixner et al., 1993</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>18.4</td>
<td></td>
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<td>Gleixner et al., 1993</td>
</tr>
<tr>
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<td>23.1</td>
<td></td>
<td></td>
<td>20.7</td>
<td></td>
<td></td>
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<td>Troughton, 1972</td>
</tr>
<tr>
<td>Cotton</td>
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<td></td>
<td></td>
<td>16.0 -17.8</td>
<td></td>
<td></td>
<td></td>
<td>Brugnoli and Lauteri, 1991</td>
</tr>
<tr>
<td>Desert plants</td>
<td>18.7 - 21.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.6 - 20.1</td>
<td>Ehleringer et al., 1992</td>
</tr>
<tr>
<td>Phragmites australis</td>
<td>20.8 ± 0.1</td>
<td>20.5 ± 0.2</td>
<td>18.3±0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M. Lauteri, A. Augusti and E. Brugnoli, unpublished</td>
</tr>
<tr>
<td>Picea Abies</td>
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<td></td>
<td>19.0</td>
<td></td>
<td></td>
<td>17.2 - 21.3</td>
<td></td>
<td>Gebauer and Schulze, 1991</td>
</tr>
<tr>
<td>Abies lasiocarpa</td>
<td>18.6 - 22.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Craig, 1953</td>
</tr>
<tr>
<td>Pinus contorta</td>
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<td></td>
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<td>Craig, 1953</td>
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</table>
lower than that of leaves (Table 1). On the other hand, root $\Delta$ is intermediate between that of leaves and that of seeds, being about 1% less than leaf $\Delta$. A further difference is observed between tuber and leaves in potato, with $\Delta$ values 1.5–2.4% lower in the former than in the latter organs (Troughton, 1972; Gleixner et al., 1993). Differences between wood and leaves in tree species are less striking, and rather variable.

There are several possible explanations for the differences in $\Delta$ observed in various anatomical compartments. One possibility is that fractionation may occur during export, xylem loading and unloading and transport of carbohydrates from photosynthetic to storage organs such as seeds and tubers. These fractionation processes might be related to glycolysis with branching at some metabolite and subsequent equilibrium isotope effects, which in turn favor the transport of one isotope species compared to the other. Different chemical composition of different organs may also be responsible for the differences in $\Delta$. For example, some organs may have a higher lipid content than others, leading to a relative depletion in $^{13}$C. Different contents in lignin (lighter) and cellulose (heavier) in the wood may explain the range of variation reported for wood $\Delta$. Similarly, differences in $\Delta$ among needles and twigs in *Picea abies* (Gebauer and Schulze, 1991), were attributed to changes in lipid, protein, hemicellulose and cellulose contents. On the other hand, differences in $\Delta$ among leaves and photosynthetic twigs in desert plants can be due to differences in diffusion limitation between organs, with twigs showing lower stomatal conductance, and hence lower $\Delta$, than leaves (Farquhar et al., 1989a; Ehleringer et al., 1992). It is known that some succulent plants fix CO$_2$ by $C_3$ photosynthesis in leaves while engaging CAM in the stems, leading to a significant shift in $\Delta$.

A further explanation of the differences in $\Delta$ among different plant parts is that some plant organs and structures may be formed at different times and developmental stages, when the contribution of diffusion and carboxylation limitation may be significantly different. For example, seeds are formed late during the plant life cycle. Often plants experience more unfavorable conditions in the late stage of development, with frequent stress imposition because of decreased soil water availability and/or increased vapor pressure deficit. This effect has been described for glasshouse- and field-grown wheat, with difference of about 5% between early-formed leaves and grain (Condon et al., 1992, 1993), attributed to drought-induced stomatal closure. Hence, during such stresses $p_t/p_a$ would be lower, and the carbon fixed at this time would be isotopically heavier. Furthermore, aging and senescence could contribute to variation in $p_t/p_a$ and $\Delta$. In addition, variation in other physiological characteristics such as mesophyll conductance to CO$_2$ diffusion during ontogeny, have been observed in some species (Loreto et al., 1994; Brugnoli et al., 1997), and this may contribute to the observed decrease in $\Delta$ during the ontogeny of plants (Scartazza et al., 1998). In addition, possible differences in dark respiration rate and in the resulting fractionation among organs may contribute to variation in $\Delta$ across different plant parts.

All the above effects could simultaneously contribute in different ways to variation in $\Delta$ of various organs. For example, it is peculiar that the smallest difference between leaf and seed $\Delta$ was observed in sunflower (Table 1), where seeds are especially rich in lipids. Hence, the isotope composition of sunflower seed could be the result of two counteracting effects: increased diffusional limitation after flowering tends to decrease the fractionation against $^{13}$C, while the high lipid content would tend to increase the $\Delta$ value of the bulk seed material.

Regardless of the underlying causes, variations in $\Delta$ among metabolites and anatomical compartments should be considered in studies devoted to quantitative analysis of physiological and ecophysiological parameters in plants.

**VI. Carbon Isotope Discrimination and the Ratio of Intercellular and Atmospheric Partial Pressures of CO$_2$**

Models of photosynthetic carbon isotope discrimination predict linear relationships between $\Delta$ and $p_t/p_a$. As already discussed, the dependency of $\Delta$ on $p_t/p_a$ is maximum in $C_3$ species, while is least in $C_4$ species, where it also depends strongly on bundle sheath leakiness.

**A. Agreement Between Models and Experimental Results**

Several experimental studies have confirmed the linear relationship between $\Delta$ and $p_t/p_a$, especially in $C_3$ plants (Fig. 2). These have integrated $\Delta$ and $p_t/p_a$.
over different time scales. Earliest reports showed linear relationships between $\Delta$ measured in the plant dry matter and $p_{/p_a}$ in several species under a wide range of experimental conditions (Winter, 1981; Farquhar et al., 1982b; Bradford et al., 1983; Downton et al., 1985; Ehleringer et al., 1985; Seemann and Christley, 1985; Flanagan and Jefferies, 1989). Although the source isotope composition was not measured in these studies, all the relationships were reasonably close to that predicted from Eq. (11), with values of $b$ ranging between 26$\%$ and 29$\%$.

The relationship between $\Delta$ and $p_{/p_a}$ has been demonstrated also in the short-term using on-line $\Delta$ measurements. Evans et al. (1986) showed linear relationships between on-line $\Delta$ and $p_{/p_a}$ in several $C_3$ and $C_4$ species. Subsequently, several authors, using on-line $\Delta$ measurements, showed results consistent with the theory, both for $C_4$ (Bowman et al. 1989; von Caemmerer and Hubick, 1989; Henderson et al., 1992; von Caemmerer et al., 1997) and for $C_3$ species (Farquhar et al., 1989a,b; Ehleringer et al., 1991, 1992; von Caemmerer and Evans, 1991; Evans et al., 1994). On-line results on $C_3$-$C_4$ intermediate species (von Caemmerer and Hubick, 1989) showed that $\Delta$ was similar to that of $C_4$ species, with significant variations depending on $CO_2$ concentration and the species. Variation among species was attributed to the presence of a glycine shuttle, either coupled or not with the $C_4$ pathway.

More recently, several authors confirmed positive relationships between bulk dry matter $\Delta$ and $p_{/p_a}$. Reasonably good agreement between the theoretical and the observed relationship was found in studies concerning wheat genotypes (Condon et al., 1990), accessions of Eucalyptus camaldulensis (Gibson et al., 1991), cotton exposed to salinity (Brugnoli and Lauteri, 1991), ozone sensitive and resistant populations of Jeffrey pine (Patterson and Rundel, 1993), the desert shrub Larrea tridentata (Rundel and Sharifi, 1993) and Festuca arundinacea (Johnson, 1993). In another study with several ecotypes of Arabidopsis thaliana (Masle et al., 1993), $\Delta$ and $p_{/p_a}$ were linearly correlated, according to Eq. (11) with $b$ values ranging between 27 and 30$\%$.

Carbon isotope discrimination in bulk plant or leaf dry matter gives an assimilation-weighted integration of $p_{/p_a}$ over the entire period during which the carbon forming that dry matter was fixed. Hence, this integration time can encompass the entire plant lifespan. Conversely, on-line $\Delta$ measurements give an instantaneous estimate of physiological events and fractionation processes associated with net photosynthetic $CO_2$ uptake. It has been demonstrated (Brugnoli et al., 1988) that carbon isotope discrimination measured in leaf soluble sugars synthesized in a diurnal course correlates with an assimilation weighted average of $p_{/p_a}$ over the entire day. The fitted regression equation gave values of $a = 3.9$ and $b = 25.5$, in agreement with the theoretical prediction, although higher $\Delta$ values would be expected for initial $C_4$ products (Brugnoli et al., 1997, see also Section V above). In the same experiment $\Delta$ of leaf starch was significantly smaller than that of sugars, although still strongly correlated with $p_{/p_a}$. The possible causes of these differences may be further fractionation processes during carbohydrate synthesis, as discussed above.

There are several other cases where the relationship between $\Delta$ measured in leaf sugars and $p_{/p_a}$ was confirmed. Figure 4 (upper panel) shows the relationships between $\Delta$ measured in leaf sugars and $p_{/p_a}$. These results were obtained on cotton plants exposed to well-watered conditions, or to drought or to 200 mM salinity stress, and in rice plants exposed to fully irrigated conditions or to drought. It is shown that $\Delta$ in leaf sugars is strongly correlated with changes in $p_{/p_a}$, in agreement with Eq. (11) with a value of $b = 25\%$. In agreement with this evidence, in a previous experiment with cotton plants exposed to continuous salinity stress, $\Delta$ in leaf sugars was correlated with $p_{/p_a}$ calculated from spot gas exchange measurements, taken during the same day on which sugars were sampled (Brugnoli and Björkman, 1992). The relationship observed was in reasonable agreement with that predicted from theory. Hence, $\Delta$ in leaf soluble sugars is a reliable indicator of changes in $p_{/p_a}$ induced by environmental stresses such as drought or salinity, imposed either in the short-term or in the long-term.

B. Complication in the Relationship Between $\Delta$ and $p_{/p_a}$

Despite experimental evidence having confirmed the relationship between $\Delta$ and $p_{/p_a}$, there are several known complications in the use of the model, especially that described by Eq. (11). As stated above, Eq. (11) does not take into account possible effects of limitation to $CO_2$ diffusion between the substomatal cavities and the sites of carboxylation, and possible fractionation processes during respiration and
When these components are substantial, the observed results will show a deviation from the predicted value. While the effect of dark respiration is thought to be close to zero (but see Duranceau et al., 1999), the fractionation associated with photorespiration may be substantial (von Caemmerer and Evans, 1991; Gillon and Griffiths, 1997). The effect of low internal conductance to diffusion, sometimes called mesophyll or wall conductance ($g_{wm}$), and the consequent drop in CO$_2$ partial pressure between the substomatal cavities and the chloroplast may be even more important in the estimation of $\Delta$ (Evans et al., 1986; Chapter 14, Evans and Loreto). Indeed, most of the deviations between observed results and $\Delta$ expected on the basis of Eq. (11), found in numerous reports, are most likely attributable to low mesophyll conductance. Certainly, this can also explain slight deviations in the relationship between $\Delta$ and $p_i/p_a$ in Fig. 4 (upper panel) and those reported previously (Brugnoli et al., 1988; Brugnoli and Björkman, 1992; Scartazza et al., 1998). A significant internal diffusion limitation may explain the deviation of the relationship from Eq. (11) observed with gap and understory plants in a tropical forest (Jackson et al., 1993), although underestimation of $\delta_a$ could also contribute to this evidence. As discussed above, $\delta_a$ in a closed tropical forest canopy can show large variations because of the relative effects of photosynthesis and respiration. Similarly, different values of $a$ and $b$ were reported for *Agropyron desertorum* growing in two different sites (Read et al., 1992). Once again, such differences may be attributed to variation in fractionation processes during liquid phase diffusion and, possibly, to differences in the source CO$_2$ composition, not measured in this experiment.

Evans et al. (1986) developed a method to estimate mesophyll conductance based on the deviation between measured on-line $\Delta$ and that calculated ($\Delta_i$) using Eq. (8) with $p_i$ set equal to $p_e$ at the same $p_i/p_e$ measured from gas exchange. This method is described in detail in Chapter 14 (Evans and Loreto, 1997).

On the basis of on-line $\Delta$ analysis it has been shown that $g_{wm}$ can be variable and quite low, particularly in woody species (Evans et al., 1986; von Caemmerer and Evans, 1991; Lloyd et al., 1992). However, the low $g_{wm}$ usually found in woody species is roughly matched by low photosynthetic capacity and by low stomatal conductance, as confirmed by the fact that there is usually a near-linear relationship between $g_{wm}$ and the assimilation rate and, consequently, between $g_{wm}$ and $g_s$ across different species. If this relationship were perfectly linear the drop in CO$_2$ pressure between $p_i$ and $p_e$ would be constant among herbaceous and woody species (Chapter 14, Evans and Loreto). However, there is significant variation in the slope of the relationship between $\Delta$ and $g_{wm}$ with consequent variation in the drop between...
aperture would therefore offset the relationship between and/or decrease the significance of in woody species.

In the above reports, on-line Δ measurements were used to determine g_m. More recently, it has been shown that the deviation between Δ and that measured in leaf sucrose can also be used to estimate g_m (Brugnoli et al., 1997; Lauteri et al., 1997; Scartazzia et al., 1998). This method has been tested in several C_3 species in different conditions, giving a good agreement with previous reports using the on-line method (see review by Evans and von Caemmerer, 1996). In agreement with previous reports, a linear relationship between assimilation rate and g_m has been observed across different species and under different environmental conditions (Brugnoli et al., 1997).

The estimate of g_m using sugar Δ gives a longer integration time of the physiological features involved, compared to that using on-line Δ. Several samples can be collected in the same day, for subsequent sugar extraction and mass spectrometric analysis. Hence, this method can be especially useful in ecophysiological applications in the field where on-line measurements are difficult. Certainly the effects of mesophyll conductance should be taken into account in studies on isotope discrimination, and especially in cases where g_m tends to be low relative to stomatal conductance. Further studies are needed to elucidate the ecological significance of g_m in terms of photosynthetic efficiency and water conservation under unfavorable conditions.

C. Other Sources of Variation in the Relationship Between Δ and p/p_a

In addition to the complication discussed above, other factors can interfere with measurements of Δ and its relationship with p/p_a. One possible problem is due to the fact that measurements of p/p_a by gas exchange can be affected by systematic errors. For example, a salinity induced decrease in Δ accompanied by an increase in p/p_a was observed in bean plants (Brugnoli and Lauteri, 1991). These results were explained by stress induced heterogeneity of stomatal conductance over the leaf surface, causing an overestimation of p/p_a as far as Δ is concerned (Terashima et al., 1988). Heterogeneous stomatal aperture would therefore offset the relationship between p/p_a and Δ and/or decrease the significance level for the best fit regression. The effect of heterogeneity of stomatal aperture on the relationship between Δ and p/p_a has been discussed in detail by Farquhar (1989). Briefly, Δ records the assimilation rate-weighted value of p/p_a while gas exchange estimates are of the conductance-weighted value. Thus heterogeneity lowers the value of p/p_a seen by Δ compared to that estimated by conventional gas exchange.

A further possible source of uncertainty in the estimation of p/p_a is given by the existence of a substantial cuticular conductance to water vapor. In conventional gas exchange studies, it is assumed that such conductance is close to zero both for CO_2 and H_2O. However, while the cuticular conductance to CO_2 should be insignificant, the conductance to water vapor may be more important. This has been shown recently in grape leaves (Boyer et al., 1997). Under stress conditions, when stomata are closed, the relative contribution of cuticular conductance to total leaf conductance may be relatively high, causing an overestimation of p_a (Meyer and Genty, 1996). Hence, this would also affect the relationship between Δ and p/p_a if the latter were not measured properly.

Differences in chemical composition and in the relative Δ of the material analyzed can also affect the relationship between Δ of bulk material and p/p_a. A correlation between Δ and p/p_a was shown for cotton plants exposed to salinity (Brugnoli and Lauteri, 1991), although the slope of the relationship was higher for Δ measured in cotton fiber (mostly cellulose) than for that in leaf dry matter. Differences in the relationship between Δ and p/p_a were also found among sun and shade leaves of Coffea arabica (Gutiérrez and Meinzer, 1994) and attributed to differences in the chemical composition or in the translocation of carbohydrates between sun and shade leaves. Furthermore, as discussed above, different metabolites or organ may show very different Δ because of difference in turnover time and in the time when the relative carbon is fixed. This effect is clearly shown in Fig. 4, where Δ in bulk leaf material (lower panel) is almost unaffected by changes in p/p_a induced by salinity or drought, while Δ in leaf sugars (upper panel) is strongly correlated with p/p_a, with a value of b=25% in reasonable agreement with Eq. (11). These results are explained by the fact that when stress is imposed leaf structural carbon is already formed and its Δ is not affected by changes in p/p_a whereas leaf soluble sugars are rapidly turned over and their Δ is strongly affected by short-term changes in p/p_a. In a previous experiment with cotton plants exposed to continuous salinity stress, Δ in leaf
sugars was slightly more sensitive to salinity-induced changes in $p_{i}/p_{a}$ than was $\Delta$ in the insoluble fraction (Brugnoli and Björkman, 1992). In agreement with these results, strong relationships were demonstrated between leaf sugar $\Delta$ and $p_{i}/p_{a}$ in different sunflower genotypes grown either under well-watered or drought conditions (Lauteri et al., 1993). The slopes of the relationships between leaf sugar $\Delta$ and $p_{i}/p_{a}$ were always higher than those for $\Delta$ in the non-soluble fraction or for bulk leaf material. The value of $b$ for the relationship between soluble sugar $\Delta$ and $p_{i}/p_{a}$ ranged between 24 to 25.5% (Lauteri et al., 1993), indicating that the observed $\Delta$ was always lower than that expected on the basis of Eq. (11), possibly because of low (finite) mesophyll conductance.

Recently, Picon et al. (1997) working on the combined effects of elevated CO$_2$ and drought on Quercus robur, reported a drought induced decrease in leaf soluble sugar $\Delta$ accompanied by a decrease in the ratio of CO$_2$ assimilation to stomatal conductance measured from gas exchange. Therefore, these results imply that $\Delta$ and $p_{i}/p_{a}$ were negatively correlated. These results were in agreement with those obtained previously on bulk leaf material $\Delta$ in Quercus petraea (Picon et al., 1996). They explained these unexpected results arguing that gas exchange measurements were not representative of the daily integrated assimilation-weighted $p_{i}/p_{a}$ because of possible significant CO$_2$ fixation early in the morning, at significantly different $p_{i}/p_{a}$ (Picon et al. 1997). Certainly, this possibility may partly explain these results. Nevertheless, possible occurrence of heterogeneity of stomatal aperture and a significant cuticular conductance to water vapor may also contribute to the inverse relationship between $\Delta$ and $p_{i}/p_{a}$.

Despite the latter reports, it is now clearly evident that carbon isotope discrimination in leaf soluble sugars is correlated with an assimilation-weighted average value of $p_{i}/p_{a}$ over a period of about one-two days. Leaf sugar $\Delta$ gives an intermediate integration time between that of bulk dry matter and on-line $\Delta$ analyses. Hence, this analysis can be extremely useful in ecophysiological studies to analyse fluctuations in environmental parameters affecting the rate of photosynthesis.

In recent experiments with several rice genotypes, it has been demonstrated that soluble carbohydrates accumulated in the stem, and specially in the uppermost internode and peduncle, have a longer turnover than that of leaf sugars. The analysis of $\Delta$ in such sugars was then compared with different integration times of $p_{i}/p_{a}$. Figure 5 shows that while peduncle sugar $\Delta$ was correlated with the $p_{i}/p_{a}$ value determined during flowering and during grain filling, the best fit regression was obtained with the assimilation-weighted value of $p_{i}/p_{a}$ over a period of about 15 days. Hence, the analysis of $\Delta$ in sugars extracted from the peduncle gives a longer integration time compared to that of leaf sugars. Differences in $\Delta$ among peduncles and other organs such as leaves

![Figure 5](image_url). Correlations between carbon isotope discrimination in Oryza sativa peduncle sugars and $p_{i}/p_{a}$ measured during flowering, during grain filling, and the assimilation weighted average of $p_{i}/p_{a}$ on a 15 day period from flowering. Measurements of $p_{i}/p_{a}$ for flowering and grain filling represent the average of measurements taken during a single day, while the weighted average is an integrated assimilation weighted average over a period of 15 days from flowering to early grain filling stage. Plants subjected to fully irrigated conditions are compared with plants subjected to drought starting from panicle-emergence. Data from A. Scartazza, M. Lauteri and E. Brugnoli (unpublished). Regression equations are: $y = 4.8 + 18.2x$, flowering; $y = 5.7 + 17.2x$, grain filling; $y = 0.91 + 24.32x$, the weighted average over 15 days from flowering to early grain filling.
were previously reported by Condon et al. (1992), but they measured \( \Delta \) of bulk peduncles rather than soluble sugars from these organs. Therefore, they obtained a relatively longer integration of \( \Delta \).

In conclusion, carbon isotope discrimination gives a good estimate of \( \frac{p_i}{p_a} \), provided that other sources of variation and anomalies discussed above are negligible. The ratio of \( \frac{p_i}{p_a} \) can be estimated at different increasing integration times, from minutes to the entire plant life cycle, using \( \Delta \) measured online, on leaf soluble sugars, on storage carbohydrates and on the bulk plant biomass, in that order. The association of all the above measurements can be useful for detecting variations in the photosynthetic performances of plants.

VII. Water Use-Efficiency, Productivity and \( \Delta \) in \( C_3 \) Species

Water use efficiency (WUE), the ratio of biomass produced to water used, is thought to be a relevant parameter in determining crop productivity, at least when water is limiting. According to Passioura (1986), crop yield (\( Y \)) is given by

\[
Y = \text{WUE} \times E \times HI,
\]

where \( E \) is the water transpired (i.e., a fraction of total evapotranspiration) and \( HI \) is the harvest index, the ratio of economical production to total biomass produced. Therefore, breeding for improved WUE may be beneficial for increasing productivity, at least in drought-prone environments. The application of \( \Delta \) to plant breeding has been previously reviewed by Hall et al. (1994).

On the basis of the positive correlation between \( \Delta \) and \( \frac{p_i}{p_a} \), carbon isotope discrimination has been proposed as a selection criterion in \( C_3 \) species for improving water-use efficiency (Farquhar et al., 1982a; Farquhar and Richards 1984). As discussed above, the \( \frac{p_i}{p_a} \) ratio represents the balance between photosynthesis and stomatal conductance to CO\(_2\). Because CO\(_2\) and H\(_2\)O share the same path for gas exchange, through stomata, any increase in \( p_a \) will improve photosynthetic CO\(_2\) uptake but will also increase the amount of water loss by transpiration. Indeed, the photosynthetic water-use efficiency (W), also called transpiration efficiency, is negatively related to \( \frac{p_i}{p_a} \). According to Farquhar et al. (1982a), W is given approximately by

\[
W = \frac{A}{E} \left( \frac{1 - P_i}{P_a} \right),
\]

where \( A \) is the rate of net CO\(_2\) assimilation and E is the transpiration rate. \( e_i \) and \( e_a \) are the water vapor pressures inside the leaf and in the surrounding atmosphere, respectively. The factor 1.6 is the ratio of the binary diffusivity of water vapor in air to that of CO\(_2\) in air.

At the whole plant level, water-use efficiency (WUE) is determined not only by the rates of CO\(_2\) uptake and water loss, but also by respiratory CO\(_2\) release and further water losses not related to photosynthetic activity. A relative amount of carbon \( \Phi \) is lost because of respiration either at night by the whole plant, or during the day by non-photosynthetic organs. In addition, a proportion (\( \Phi_w \)) of total water loss is independent of CO\(_2\) uptake, such as water lost at night through stomata or by other organs and cuticular transpiration during daytime. Hence, according to Farquhar et al. (1989a), WUE is given by

\[
\text{WUE} = \frac{A}{E} \left( \frac{1 - \Phi_e}{1 + \Phi_e} \right).
\]

Combining Eqs. (16) and (17) with Eq. (8) it is evident that a negative relationship is expected between \( \Delta \) and WUE in \( C_3 \) plants, given by

\[
\text{WUE} = \frac{P_a \left( \frac{b - d - \Delta}{b - a} \right) (1 - \Phi_e)}{1.6(e_i - e_a)(1 + \Phi_w)},
\]

where the term \( d \) summarizes all possible fractionation processes associated with liquid phase diffusion, photorespiration and respiration (Farquhar et al., 1989a). In \( C_4 \) species, based on the negative relationship between \( \Delta \) and \( p_i/p_a \), the correlation between \( \Delta \) and WUE should be positive as confirmed recently in sorghum (Henderson et al., 1998).

Experimental results on several \( C_3 \) species (see review by Hall et al., 1994) showed negative correlations between \( \Delta \) and WUE, in reasonable agreement with theoretical expectations. More recently, similar results have been confirmed on tree species such as *Eucalyptus camaldulensis* (Hubick and Gibson, 1993) and *E. globulus* (Osorio and Pereira, 1994), *Pinus pinaster* and *Quercus petraea*.
In such experiments, negative correlations between $\Delta$ and WUE were either genetically or environmentally induced, or both. Environmentally induced increases in WUE, as under drought and other stresses, are expected to be accompanied by decreases in $\Delta$, because under drought, changes in $p_i/p_s$ and WUE will be largely due to stomatal closure.

Genotypic variation in $\Delta$ associated with variation in WUE has been explored both in controlled environments and under field conditions. Studies on several species including wheat, barley, rice, bean, peanut, cowpea, alfalfa and sunflower have shown genetic variation in $\Delta$ (see review by Hall et al., 1994). Genetic variation in $\Delta$ associated with variation in stomatal conductance has also been reported in bean (Ehleringer, 1990), *Gossypium barbadense* (Lu et al., 1996), *Pseudotsuga menziesii* and *Pinus ponderosa* (Zhang and Marshall, 1995). In addition, it has been demonstrated in several species that $\Delta$ is under genetic control and heritable, although variable results have been reported for different species and environmental conditions (Hall et al., 1994). Subsequently, it has been shown (Ismail and Hall, 1993) that inheritance of $\Delta$ and WUE is nuclear in cowpea, and no maternal effects were observed. No conclusive results were reported in this study about the possible presence of dominance. Low $\Delta$ appeared to show partial dominance in a pot experiment, while in the field high $\Delta$ was partially dominant. These authors attributed the contrasting evidence to differences in rooting conditions, but in a subsequent study they failed to demonstrate unequivocal correlation between $\Delta$ or WUE and xylem ABA concentration (Ismail et al., 1994), which they thought should represent the main hormonal root signal. Certainly many other factors may influence these results.

Genetic control of $\Delta$ has been demonstrated studying the association between genetic markers and variation in $\Delta$. Martin et al. (1989) showed that most of the variation in $\Delta$ between two tomato species was associated with three restriction fragment length polymorphisms (RFLPs). Subsequent RFLP analysis of $\Delta$ in *Arabidopsis thaliana* (Masle et al., 1993) was hindered by genotype x environment interactions. Variation in $\Delta$ was related to genetic variation in 21 isozyme loci among Turkish populations of *Castanea sativa* (Villani et al., 1992). The variation in $\Delta$ among chestnut populations was mostly attributable to changes in photosynthetic capacity and mesophyll CO$_2$ transfer conductance (Lauteri et al., 1997). Quantitative trait loci (QTL) analysis has been also used to study WUE and $\Delta$ in trees and crop species (Guehl J.M., personal communication, Mian et al., 1998).

Using wheat-barley disomic chromosome addition lines and wild barley populations, Handley et al. (1994) concluded that chromosome 4 controls at least some of the observed variation in $\Delta$ in barley. On the other hand, studies with chromosome substitution lines and dimonotelosomic lines in wheat indicated that all chromosomes were influencing $\Delta$, suggesting that many genes are involved in the control of this trait (Hall et al., 1994; Ehdaie and Waines, 1997). Certainly new molecular techniques becoming available will greatly improve the knowledge about the genetic control of $\Delta$ and WUE.

The negative relationships between $\Delta$ and WUE reported in various C$_4$ species and the genetic control of these traits are encouraging for application in breeding programs for increased yield and yield stability, at least under water-limiting conditions. However, early reports showed that $\Delta$ may be directly related to biomass production or grain yield in wheat under field conditions (Condon et al., 1987). Similar results were reported for wheat (Ehdaie et al., 1991; Read et al., 1991a; Araus et al., 1993; Sayre et al., 1995), barley (Craufurd et al., 1991; Acevedo, 1993), and other grasses (Johnson et al., 1990). The positive relation between yield in good environments and $\Delta$ in C$_4$ grasses (and negative in the C$_3$ grass *Zea mays*) was reviewed by Farquhar et al. (1994). On the other hand, negative correlations between $\Delta$ and grain yield were reported for lines of wheat near-isogenic for dwarfing genes (Ehdaie and Waines, 1994). Similar results were also reported for the woody shrub *Chrysothamnus nauseosus* where biomass production and $\Delta$ were inversely related (Donovan and Ehleringer, 1994b). In other studies, the sign of correlations between $\Delta$ and yield or WUE were dependent on environmental conditions (e.g., irrigated or drought, Read et al., 1991b; Ehdaie and Waines, 1994). Similarly, a negative correlation between $\Delta$ and tree height has been found in *Picea mariana* on a dry site, whereas no correlation was observed in sites characterized by higher water availability (Flanagan and Johnsen, 1995). In some of the results reported, the correlation between $\Delta$ and yield was environmentally induced, while the genotypic correlation within species was non-significant or
with different slopes (Morgan et al., 1993, Johnson and Basset, 1991).

There are several possible explanations of the conflicting results found in the literature. Under non-limiting water conditions, an improved WUE and low $\Delta$ would not be necessarily beneficial (Condon and Richards, 1993). Indeed, when the difference in $\Delta$ is mostly due to changes in stomatal conductance, genotypes with high $\Delta$ would show high conductance and photosynthesis rates and consequently high yield. Thus, a positive correlation between $\Delta$ and grain yield does not imply a positive correlation between $\Delta$ and WUE. Indeed positive correlations between $\Delta$ and yield accompanied by negative correlations between $\Delta$ and WUE have also been observed (Johnson et al., 1990; Brugnoli et al., 1997). Hence, under irrigated conditions WUE will not generally be an essential feature for high or stable yield. On the other hand, when differences in $\Delta$ are due to changes in photosynthetic capacity (Hubick et al., 1986), any improvement in WUE is expected to be beneficial, both under irrigated and drought conditions.

A further complication in the correlation between $\Delta$ and WUE is represented by the canopy structure, which may influence soil water losses and boundary layer resistance (reviewed by Farquhar et al., 1989a). In addition, the vapor pressure difference between leaf and atmosphere is not an independent variable and may be affected by changes in conductance and in leaf temperature. This may be relevant when comparing both environmental and genetic effects on $\Delta$ (Farquhar et al., 1989a; Ehleringer et al., 1992; Comstock and Ehleringer, 1993; Condon et al., 1993; Brugnoli et al., 1997). Further insights in this subject are expected from the study of oxygen isotopes in plants (Farquhar et al., 1997).

Other physiological, morphological and developmental parameters may be directly or indirectly related to $\Delta$, influencing the relationship between $\Delta$ and WUE or yield. It has been shown that changes in carbon allocation patterns among roots, leaves and stems and relative growth rate are associated with changes in $\Delta$ in sunflower and other species (Farquhar et al., 1989a; Virgona et al., 1990; Virgona and Farquhar, 1996; Scartazza et al., 1998). Increased allocation to root may be beneficial for taking up water from deeper soil layers during drought. Root length density was positively correlated with $\Delta$ in bean genotypes (White et al., 1990). A negative correlation has also been reported between $\Delta$ and specific leaf area in peanut (Wright et al., 1994). In addition, it has been shown that early flowering genotypes show higher $\Delta$ than late flowering ones (Hall et al., 1994). The correlation between $\Delta$ and earliness may represent a disadvantage when selecting drought ‘tolerant’ genotypes on the basis of $\Delta$, because early flowering is a major drought ‘avoidance’ mechanism. The correlation is thought not to be causal (Hall et al., 1994).

Often, yield is strongly dependent on water availability during specific developmental stages, such as flowering. At this stage, drought is especially detrimental because it causes flower sterility and consequent yield losses. It is expected that an increased WUE during this stage may be useful for conferring ‘drought tolerance’. In several studies on different upland rice genotypes, it has been demonstrated that $\Delta$ measured in ethanol soluble sugars (i.e., mainly sucrose) extracted from the upper internode and peduncles sampled 15 days after panicle emergence flowering, was negatively related to grain yield (B. Pinheiro, R.B. Austin, E. Brugnoli, A. Scartazza and M. do Carmo, unpublished). This genetic correlation was found both under irrigated and drought conditions, when drought was imposed at flowering. This correlation is explained by the relationship between $\Delta$ in stem sugars and the average of $p_v/p_a$ over a period of about two weeks (Fig. 5), suggesting that this $\Delta$ value gives an estimate of WUE during the period of flowering and early grain filling. Hence, low $\Delta$ during this stage would imply higher WUE at leaf level, which in turn may be beneficial for flower fertility. On the other hand, a short period of drought at this stage does not affect significantly the $\Delta$ value of bulk dry matter, despite possible dramatic effects on grain yield. This may explain why correlations between bulk dry matter $\Delta$ and yield were less significant (E. Brugnoli, A. Scartazza, M.C. Monteverdi, M. Lauteri and A. Augusti, unpublished). Hence, the analysis of $\Delta$ in internodes and peduncle carbohydrates may be used to assess WUE during crucial periods for fertility and productivity. This method represents a significant improvement with respect to the use of $\Delta$ analysis in peduncle dry matter introduced by Condon et al. (1992).

Other possible mechanisms influencing plant WUE and $\Delta$ which need to be considered include leaf movements. Leaf rolling may affect plant WUE and $\Delta$ in rice (Dingkuhn et al., 1991). Similarly, active paraheliotropic leaf movements in bean may influence the leaf energy balance and temperature, affecting
the relationship between $\Delta$ and WUE (Ehleringer et al., 1991). Such mechanisms are expected to increase WUE. In addition, hormone signaling (e.g. ABA) between root and shoot may also play a role in regulating WUE. Nevertheless, the effects of either genetic or environmental variations in ABA content on $\Delta$ are not clear (Read et al., 1991a; Ismail et al., 1994; A. Scartazza and E. Brugnoli, unpublished). Further studies should investigate the complex effects of ABA on $\Delta$, yield and drought tolerance.

In spite of the large amount of effort in the study of $\Delta$ in relation to water-use efficiency and productivity, our understanding of the complex mechanisms influencing these relationships is still rudimentary. More information is needed about interactions between roots and shoots, with special emphasis on root signals. Furthermore, the study of temporal variation in WUE during special stages of development, such as flowering, deserves more attention. New insight on this subject is expected from the study of carbon isotope discrimination in carbohydrate pools, which can give information on WUE at different integration times.

VIII. Carbon Isotope Discrimination and Physiological Ecology of Photosynthesis

The analysis of plant carbon isotope ratios has become a useful tool for assessing photosynthetic performance and relative water-use efficiency in ecophysiological and ecological studies. Recently this has involved the role of relative changes in photosynthetic capacity and stomatal conductance in the distribution of species, in population dynamics within species, and in adaptation to the environment.

Variations in $\Delta$ among plant populations in relation to the area of origin and to environmental parameters such as water availability, temperature, and altitude have been studied. The information available indicates a complex relationship between $\Delta$ and adaptation to stress conditions. For example, one would expect that populations of plant adapted to dry conditions have low $\Delta$ and high WUE. However, studies conducted with several populations and species grown in common gardens have shown that this may not always be the case. In *Nothofagus* species a negative correlation between $\Delta$ and summer rainfall in the area of origin was reported (Read and Farquhar, 1991). These results would imply that species from regions with summer-dry climates have lower WUE.

Other adaptive mechanisms, such as high capacity for water uptake and differential stomatal sensitivity, were invoked to explain these results. Recently, similar results have been demonstrated in chestnut populations grown in a common field, with populations originating from the driest environment showing higher $\Delta$ than those collected in relatively wet sites (Lauteri et al., 1997). Similar results were observed in *Faidherbia albida* provenances (Roupasard et al., 1998). In contrast, positive correlations between $\Delta$ and mean annual precipitation and seasonality of precipitation were reported for populations of 14 species of *Eucalyptus* (Anderson et al., 1996, but see Brodribb and Hill, 1998). In a different experiment in situ, i.e. combining genetic and direct environmental effects, a positive correlation between $\Delta$ and average rainfall was also reported across 348 species growing along a rainfall gradient, corresponding to an aridity gradient, in Southern Queensland (Stewart et al., 1995). A similar positive relationship between $\Delta$ and rainfall, but limited to the most arid conditions (rain below 475 mm) has been recently reported among trees along a transect through Northern Australia (Schulze et al., 1998). These results indicate that populations from more arid environment would be more efficient in the use of water. In a comparison of species of the Mediterranean macchia, it has been reported that evergreen species, which rely mostly on rain-water, have lower $\Delta$ and higher WUE than deciduous species depending almost exclusively on ground-water (Valentini et al., 1992). Similar results were reported among Larches and sympatric evergreen species (KloeppeI et al., 1998).

The results available on this subject reflect different strategies for adaptation and survival in dry environments. It is possible that the best compromise between water conservation and photosynthetic CO$_2$ assimilation is dependent on the extent and duration of drought periods experienced. The lack of a simple correlation between WUE and growth and survival was also evident in natural shrub populations growing in arid and semi-arid environments (Donovan and Ehleringer, 1994a). In addition, in an elegant experiment Ehleringer (1993) has shown in *Encelia farinosa* populations that tradeoffs between conditions favoring high- or low-$\Delta$ genotypes may exist in these desert species. Genotypes with low $\Delta$ may be favored under long-term drought or high competitive pressure, while genotypes with high $\Delta$ and high photosynthetic rates will benefit most of period with high availability of resources.
Variation in $\Delta$ and WUE have been reported in relation to size and age classes in natural populations (Donovan and Ehleringer, 1992). Discrimination is also variable with life forms, with annual species discriminating more than perennials, and grasses discriminating less than forbs (Smedley et al., 1991). Sex-related differences in $\Delta$, have been reported for the dioecious tree species *Acer negundo* (Dawson and Ehleringer, 1993). Male trees showed lower stomatal conductance, photosynthesis rate, $p_v$, and $\Delta$, compared to female trees. This pattern of variation explains the sex-biased distribution in box elder, with male trees dominating droughted habitats and female trees dominating wet, streamside habitats. This pattern of distribution would likely optimize the pollination efficiency and the cost for reproduction. The opposite pattern was observed in *Phoradendron juniperinum*, a dioecious xylem-tapping mistletoe, with males showing higher photosynthesis rate and lower $\Delta$, and dominating habitat with higher resource availability (Marshall et al., 1993). This may be explained by the different ecological distribution of epiphytes, and a different cost of reproduction in males in this species.

From an evolutionary point of view, it is clear that selective pressure may favor divergent genotypes and photosynthetic behaviors. Plants with low $\Delta$ and higher WUE may be favored in dry environments. On the other hand, plants with high $\Delta$ and high photosynthetic productivity may be favored under conditions (spatially or temporally distinct) of non-limiting resources. Hence plants with diverging $\Delta$ values may coexist in the same environment, increasing species plasticity and likelihood of survival.

**IX. Concluding Remarks**

Carbon isotope discrimination has contributed significantly in improving our understanding of photosynthesis and water-use efficiency in plants. More information is becoming available on fractionation processes associated with carbon metabolism and especially secondary metabolism. This will also aid in improving carbon isotope discrimination models, taking into account fractionation processes subsequent to photosynthetic carboxylation. Further studies will elucidate fractionation processes during decarboxylations to understand the fate of carbon originating from $\beta$-carboxylations.

Carbon isotope discrimination has been increasingly used in global and ecosystem studies, in order to scale-up the understanding of the regulation of photosynthesis. Certainly, significant advancements are expected from the joint studies of fluxes of $^{18}$O/$^{16}$O and H/D isotopes in addition to stable carbon isotopes in ecosystem studies. Recent technological advancements will greatly help such studies.

There is also an increasing interest in the study of carbon isotope discrimination in trees. However, there is still a gap of knowledge in this field, which is expected to be overcome in future studies. In particular, the study of mesophyll conductance in these plants deserves more attention for understanding the mechanisms and the significance of low mesophyll conductance.

The use of new molecular approaches is expected to give further insight and new possibilities to study the genetic control of $\Delta$, photosynthetic efficiency and WUE and to assist the use of carbon isotope discrimination in breeding programs for improved crop yield in drought regions.

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Chapter 18

C₄ Photosynthesis: Mechanism and Regulation

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Summary

The C₄ photosynthetic process is a combination of biochemical, anatomical and genetic specialization which concentrates CO₂ at the site of carbon fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase. This essentially suppresses photorespiration and allows carbon fixation to proceed at near CO₂ saturation. In this review we summarize the three diverse biochemical mechanisms underlying C₄ photosynthesis, describe the regulation of individual enzymes in the C₄ pathway, discuss the integrated regulation of C₄ photosynthesis and how these regulatory processes respond to environmental conditions.

I. Introduction

C₄ photosynthesis is a unique blend of modified biochemistry, anatomy and organelle function designed to avoid the worst outcomes of Rubisco’s oxygenase reaction and the associated process of photorespiration. This elaboration of the conventional PCR cycle functions to concentrate CO₂ at the site of Rubisco operation, thereby suppressing the oxygenase reaction and eliminating the need for the photorespiratory cycle.

It is several years since general aspects of the biochemistry and physiology of C₄ photosynthesis were reviewed (Edwards and Walker, 1983; Hatch, 1987). However, a comprehensive account of C₄ biology is currently being prepared for publication (Sage and Monson, 1999). The main purpose of the present chapter is to review developments in our

References

understanding of the regulation of C₄ photosynthesis. However, to provide a framework for this discussion we will briefly describe the biochemical processes operating to transfer CO₂ to bundle sheath cells and emphasize the more recent advances in the understanding of these mechanisms. Subsequent chapters will consider in detail the metabolite transport processes that are an integral part of the C₄ mechanism as well as developmental and ecophysiological aspects of this process.

II. Mechanism and Function of C₄ Photosynthesis

A. Mechanism

The biochemistry of C₄ photosynthesis and its function are intimately linked with a highly specialized leaf anatomy. Leaves of C₄ plants contain two distinct types of photosynthetic cells (Fig. 1). These are usually arranged radially around vascular tissue in two adjacent layers, the outer layer being termed mesophyll cells and the inner layer, bundle sheath cells. A critical and universal feature of this type of anatomy is that Rubisco and the PCR cycle, responsible for the ultimate assimilation of CO₂, occur only in the inner bundle sheath cell layer.

In all C₄ plants CO₂ is initially fixed in mesophyll cells via PEP carboxylase (PEPC). The inorganic carbon species used by this enzyme is HCO₃⁻ so that the first reaction is, in fact, the carbonic anhydrase-mediated hydration of CO₂ (Hatch and Burnell, 1990). The oxaloacetate formed in this carboxylation reaction is converted to larger pools of the acids malate and aspartate and one or other of these acids then diffuses to the bundle sheath cells where it is decarboxylated to release CO₂. This CO₂ is fixed by Rubisco and the PCR cycle and the 3-carbon compound remaining from the decarboxylation reaction is returned to mesophyll cells to serve as a precursor for the regeneration of PEP.

A most unexpected outcome in the story was the realization that three quite distinct biochemical mechanisms operate to concentrate CO₂ in the bundle sheath cells of different C₄ species. These mechanisms are outlined in Fig. 2. The key feature distinguishing these three biochemical options for C₄ photosynthesis is the mechanism of acid decarboxylation in the bundle sheath cells. These decarboxylases, NADP-ME, NAD-ME and PEPCK (see later sections) function in the ‘NADP-ME-type’, ‘NAD-ME-type’ and ‘PCK-type’ mechanisms, respectively, described in Fig. 2. A detailed account of these schemes is provided in an earlier review (Hatch, 1987). These mechanisms appear to account for photosynthesis in the majority of C₄ plants. However, it should be emphasized at the outset that some species may use processes which are intermediate between these ‘classical’ mechanisms. Examples of such intermediacy will be mentioned.

The first of these options to be recognized and resolved was the NADP-ME-type which accounts for photosynthesis in grass species like maize, sugar cane, and sorghum as well as several dicotyledonous species. In this process OAA formed by PEP carboxylase is reduced to malate in mesophyll chloroplasts by NADP-MDH and the malate is then transferred to bundle sheath cells (Fig. 2). After entering the chloroplasts, malate is decarboxylated by NADP-ME, generating both CO₂ and NADPH. The CO₂ is fixed by Rubisco and the PCR cycle, which are specifically located in these cells, and the

Abbreviations: CAM – Crassulacean acid metabolism; FBP – fructose 1,6-bisphosphate; G6P – glucose 6-phosphate; NADP-MDH – NADP malate dehydrogenase; NAD-ME – NAD malic enzyme; NAD-ME-type – NAD malic enzyme-type; NADP-ME – NADP malic enzyme; NADP-ME-type – NADP malic enzyme-type; OAA – oxaloacetate; PCK-type – phosphoenolpyruvate carboxykinase-type; PCR cycle – photosynthetic carbon reduction cycle; PEP – phosphoenolpyruvate; PEPCK – phosphoenolpyruvate carboxylase; PEPC-PK – phosphoenolpyruvate carboxylase protein kinase; PGA – 3-phosphoglycerate; PPDK – pyruvate, Pi dikinase; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase
Fig. 2. Path of carbon assimilation and the inter- and intracellular location of reactions for the three biochemically distinct subgroups of C₄ species. The main path of carbon flow and associated metabolite transport processes are indicated by heavy arrows. The enzymes involved, indicated by the numbers in brackets, are: 1, phosphoenolpyruvate carboxylase; 2, NADP malate dehydrogenase; 3, NADP malic enzyme; 4, pyruvate Pi dikinase; 5, 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase; 6, aspartate aminotransferase; 7, NAD malate dehydrogenase; 8, NAD malic enzyme; 9, alanine aminotransferase; 10, phosphoenolpyruvate carboxykinase; 11, mitochondrial NADH oxidation systems. For the PCK-type mechanism the PGA/dihydroxyacetone-phosphate shuttle would also operate between cells (as shown for other types) and the cycling of amino groups between mesophyll and bundle sheath cells involves alanine and alanine aminotransferase (see text).
pyruvate remaining after decarboxylation is returned to the mesophyll chloroplasts and converted to the \( \text{CO}_2 \) acceptor PEP by pyruvate, P dikinase (PPDK). It should be noted that with this mechanism half the NADPH necessary for the subsequent reduction of PGA to triose phosphate is generated in the bundle sheath chloroplasts during malate decarboxylation. Particularly in those species where Photosystem II is almost totally absent from the bundle sheath chloroplasts, the remaining PGA must be transferred to mesophyll cells for reduction. Most NADP-ME-type grasses have very little Photosystem II activity in bundle sheath cells (Edwards et al., 1976; Chapman et al., 1980). However, there is substantial Photosystem II activity in the bundle sheath cells of some NADP-ME-type dicotyledons (Meister et al., 1996) and this is associated with increased transport of aspartate to bundle sheath cells which, of course, would not involve the transfer of reducing power.

A wide range of grasses and dicotyledonous species from various genera use the NAD-ME-type mechanism for \( \text{C}_4 \) photosynthesis. In this mechanism, the major \( \text{C}_4 \) acid transported to bundle sheath cells is aspartate (Fig. 2). There, aspartate enters the mitochondria together with 2-oxoglutarate and is converted through aspartate aminotransferase to OAA. The OAA is then reduced to malate and the malate decarboxylated by NAD-ME, specifically located in these mitochondria. It is important to note that where aspartate is the \( \text{C}_4 \) acid entering mitochondria, these latter two steps are coupled through an NAD/NADH cycle. However, an option under certain conditions is that malate from mesophyll cells may enter the mitochondria and then be decarboxylated through the coupled action of malate dehydrogenase and NAD-ME. This alternative route may be limited by the concentration of aspartate to bundle sheath cells which, of course, would not involve the transfer of reducing power (Agostino et al., 1996).

As with the NADP-ME-type mechanism, the pyruvate formed during the decarboxylation of malate by NAD-ME is returned to mesophyll cells where it serves as a precursor for the regeneration of PEP. However, in the NAD-ME-type species the \( \text{C}_3 \) compound actually transferred is apparently alanine. By this means, 2-oxoglutarate is regenerated in bundle sheath cells to sustain aspartate conversion to OAA and the deamination of alanine in mesophyll cells, giving glutamate as well as pyruvate, would be stoichiometrically linked with the continuing generation of aspartate (Fig. 2). As with NADP-ME-type species, the leaf’s capacity for converting PGA to triose-P (by PGA kinase and NADP glyceraldehyde 3-P dehydrogenase) is distributed about equally between mesophyll and bundle sheath cells. From this it is assumed that a substantial part of the PGA generated in bundle sheath cells is transferred to mesophyll cells for reduction, thus ensuring that both cell types contribute to the Photosystem II-based generation of the required NADPH.

So far, the PCK-type mechanism has been identified only in a range of grass species. Although distinguished by the operation of PEP carboxykinase (PEPCK) for decarboxylation it will be seen that NAD-ME contributes to the decarboxylation of \( \text{C}_4 \) acids while serving to generate ATP in mitochondria to drive the PEPCK reaction. Thus, it is visualized that both aspartate and malate contribute to the transfer of \( \text{C}_4 \) acids to bundle sheath cells (Fig. 2). This view of the PCK-type mechanism was developed in a series of studies over the last decade (Burnell and Hatch, 1988; Hatch and Carnal, 1992; Carnal et al., 1993; Agostino et al., 1996).

In PCK-type species, OAA decarboxylated in the cytosol of bundle sheath cells by PEPCK is derived from aspartate transported from mesophyll cells. The PEPCK reaction uses ATP synthesized in the mitochondria during coupled oxidation of NADH generated by NAD-ME. PEP formed during this OAA decarboxylation is presumably transported back to mesophyll cells and used directly for the further assimilation of \( \text{CO}_2 \) by PEPC. An option, which can at least be demonstrated with isolated bundle sheath cells (Carnal et al., 1993), is that OAA generated in the bundle sheath cells may enter the mitochondria and then be decarboxylated through the coupled action of malate dehydrogenase and NAD-ME. This alternative route may be limited by the concentration of OAA that prevails in vivo.

To generate ATP for the PEPCK reaction, malate must be transported from mesophyll cells to the bundle sheath mitochondria (Fig. 2). Even assuming a near theoretical yield of three ATP generated through coupled NADH oxidation, this would still require one mole of malate transported and decarboxylated by NAD-ME for each three moles of OAA decarboxylated by PEPCK. In some species the potential for malate decarboxylation and respiratory oxidation of NADH by these bundle sheath mitochondria is greater than this. This has led to the suggestion that NAD-ME may make a greater contribution to \( \text{C}_4 \) acid decarboxylation under some circumstances (Carnal et al., 1993; Agostino et al., 1996). Where malate is decarboxylated by NAD-
ME, the product pyruvate would be converted back to PEP after transfer to the mesophyll chloroplasts. It should also be noted that available evidence clearly indicates a key role for alanine and alanine aminotransferase in maintaining the amino group balance between mesophyll and bundle sheath cells (Fig. 2). However, the exact stoichiometry of these reactions and their relation to the aspartate/PEP and malate/pyruvate cycles is uncertain.

The options for $C_4$ photosynthesis described in Fig. 2 involve a remarkable array of metabolite transport processes into and out of chloroplasts and mitochondria. There is, in addition, a variety of fluxes required between mesophyll and bundle sheath cells, all being required to proceed at rates matching overall rate of photosynthesis. These will be considered in Chapter 19 (Leegood).

### B. Function and Efficiency

Viewed simply, the function of the special reactions of $C_4$ photosynthesis is to transfer CO$_2$ from mesophyll to bundle sheath cells. In fact, as already noted in the Introduction, the specific purpose is to concentrate CO$_2$ in bundle sheath cells with a view to eliminating the Rubisco-catalysed oxygenase reaction and associated photorespiration. For normal $C_3$ plants in air the oxygenase reaction proceeds at about half the rate of the carboxylation of RuBP. This results in a large decline in efficiency compared with the situation where there is negligible oxygenase. Measured in terms of quantum yield for CO$_2$ fixation, the decline is from about 0.08 mol CO$_2$ fixed/mol quanta to 0.05 at 30 °C, that is about 40%. By concentrating CO$_2$ in bundle sheath cells the oxygenase reaction and associated photorespiration are very largely eliminated in $C_4$ plants under normal conditions. However, there is an energetic cost in concentrating this CO$_2$—essentially equivalent to the 2 ATP consumed in the PPDK reaction (somewhat more complicated in PCK-type species, see Hatch, 1987). Hence, the quantum yields for $C_4$ plants, in the range from 0.06 to 0.07 mol CO$_2$/mol quanta for most $C_3$ plants at 30 °C (Ehleringer and Pearcy, 1983), are higher than the values for $C_3$ plants in air but less than the values for the latter plants measured in the absence of photorespiration. One of the factors that contributes to reducing the efficiency of photosynthesis in $C_4$ plants is the extent to which CO$_2$ leaks back to mesophyll cells instead of being refixed. This, and other aspects of the function and efficiency of $C_4$ photosynthesis, have been discussed in more detail previously (Farquhar, 1983; Hatch, 1987, 1992a, 1995; Furbank et al., 1990b; Ehleringer and Monson, 1993).

Largely as the result of almost abolishing photorespiration under normal ambient conditions, and the operation of Rubisco at or near its $V_{max}$, $C_4$ plants are capable of higher rates of photosynthesis at both limiting and saturating light compared with $C_3$ plants (Hatch, 1992a,b). However, these advantages are only apparent at higher temperatures, generally above 20–25°C, the result of photosynthetic efficiency decreasing in $C_3$ plants as temperature increases (Ehleringer and Pearcy, 1983). The main basis of this effect is a disproportionate increase in Rubisco oxygenase activity relative to carboxylase activity as temperature rises, resulting in a greater increase in photorespiration relative to photosynthesis (Jordan and Ogren, 1984). A related feature of $C_4$ plants is higher water-use efficiency; at higher temperature they average about twice the carbon assimilation per unit of water loss compared with $C_3$ plants. Thus, at higher temperature and light the $C_4$ photosynthetic option provides plants with the potential for rapid growth and this can be manifested in terms of record rates of dry matter production (Hatch, 1992a,b). However, many $C_4$ species choose the option of conserving water instead, explaining their dominance in more arid situations (Stowe and Teeri, 1978; Hattersley, 1992). These aspects are considered in more detail in Chapter 22, Monson and Rawsthorne.

### III. Regulation of Individual Enzymes

This section will deal with the details of the mechanisms operating to regulate some key enzymes of $C_4$ photosynthesis. For other enzymes implicated in the $C_4$ process, including the aminotransferases, adenylate kinase and pyrophosphatase, there is no evidence for significant regulation. Later sections will deal with the integrated regulation of $C_4$ photosynthesis and responses to environmental changes.

#### A. Phosphoenolpyruvate carboxylase

PEPC catalyses the primary photosynthetic carboxylation reaction in $C_4$ plants yielding the initial $C_4$ acid of the $C_4$ cycle in the mesophyll cytosol:
PEP + HCO$_3^-$ → OAA + Pi

The enzyme plays a key role in regulation of the C$_4$ cycle and its coordination with the C$_3$ cycle. Various isoforms of the enzyme are ubiquitous in plants as well as in many microorganisms. However, the isoform of PEPC involved in C$_4$ photosynthesis is specifically synthesized in leaves in response to light and achieves very high levels of activity, between 15 to 30 times the activity in C$_3$ leaves and about five times the maximum rate of photosynthesis when assayed in vitro under optimal conditions. This clearly indicates that, in vivo, the enzyme is under constant ‘restraint’ which is effected via allosteric metabolite- and covalent modification regulatory mechanisms. Similarly, the form of the enzyme involved in CAM photosynthesis is thought to be closely regulated in a diurnal cycle and the regulatory mechanisms may be similar. PEPC is arguably the most-studied enzyme of those involved in C$_4$ photosynthesis and the properties, mechanism and regulation of the enzyme in plants have been reviewed in detail on a number of occasions (O’Leary, 1982; Andreo et al., 1987; Stiborova, 1988; Jiao and Chollet, 1991; Lepiniec et al., 1994; Gadal et al., 1996; Chollet et al., 1996).

Activity of PEPC is subject to activation or inhibition by metabolites and the possible physiological relevance of this was recognized in early studies using crude or partially-purified extracts of C$_4$ plants (Huber and Edwards, 1975; O’Leary, 1982). Activation occurs with phosphorylated metabolic intermediates, particularly G6P, triose-phosphate and fructose 6-phosphate, with G6P often considered the most effective (Doncaster and Leegood, 1987; Gao and Woo, 1996a). The enzyme from monocot C$_4$ species (but not dicots) is also activated by certain amino acids, notably glycine, serine, and alanine (Nishikido and Takanashi, 1973; Doncaster and Leegood, 1987; Gao and Woo, 1996a). The enzyme from monocot C$_4$ species (but not dicots) is also activated by certain amino acids, notably glycine, serine, and alanine (Nishikido and Takanashi, 1973; Doncaster and Leegood, 1987; Gao and Woo, 1996a). PEPC activity is inhibited by the C$_4$ acids malate and aspartate which is of obvious importance in the present context since these are metabolites of the C$_4$ cycle. With the purification of the enzyme, initially from maize (Uedan and Sugiyama, 1976) and subsequently from a range of C$_4$ species, the oligomeric structure was determined, allosteric regulatory properties were confirmed and responses to pH and metabolites examined in greater detail (O’Leary, 1982; Andreo et al., 1987; Stiborova, 1988). The enzyme occurs as a tetramer of 109 kD subunits, but it has more recently been recognized that these subunits may be easily degraded by N-terminal deletion to approximately 105 kD products. This can be prevented by protease inhibitors, particularly chymostatin (McNaughton et al., 1989; reviewed by Chollet et al., 1996). Since the N-terminal region contains the regulatory phosphorylation site (see below), the significance of some earlier conclusions regarding regulatory properties may be doubtful. In vitro, maximum activities are observed around pH 8, the response to PEP concentration is usually hyperbolic, and at this pH the inhibitory effects of C$_4$ acids and stimulatory effects of phosphorylated metabolites are decreased. At the more physiologically relevant pH, around 7, activity is lower, cooperative binding of PEP occurs, and strong allosteric effects of activators and inhibitors are observed (Huber and Edwards, 1975; O’Leary, 1982; Andreo, 1987; Stiborova, 1988). Activation by G6P mainly alters the K$_m$(PEP) whereas glycine modifies both K$_m$(PEP) and V$_{max}^{\text{meas}}$, with these types of activators considered to operate at separate sites. Malate inhibition appears competitive with PEP at pH 7 but not at pH 8. Whether malate acts at the substrate site or by conformational changes resulting from its binding at another site is not resolved. Binding site studies with chemical modification reagents earlier indicated that various residues may be involved in PEP and metabolite binding, but few of these have been assigned to specific domains (Jiao et al., 1990) and, more recently, the earlier findings have been questioned (Gao and Woo, 1996b). Specific site-directed mutagenesis and domain swapping studies are in progress which should clarify functional regions and residues of the protein. A three dimensional molecular structure is yet to emerge.

Findings more than a decade ago (Budde and Chollet, 1986; Huber and Sugiyama, 1986; Doncaster and Leegood, 1987) that the properties of the enzyme in leaf extracts were modified in response to previous light or dark pre-treatments, along with the observation that phosphorylation of the enzyme could occur, stimulated intensive efforts to characterize regulatory phosphorylation mechanisms. When measured under sub-optimal, but probably more physiological, conditions of low PEP concentration and low pH, enzyme in extracts from illuminated leaves is less sensitive to malate inhibition and activated to a greater extent by phosphorylated metabolites, than enzyme from darkened leaves. Both enzyme forms show similar activity under optimal in vitro conditions. It is now generally accepted that
phosphorylation of the enzyme plays an important regulatory role in relation to light-dark changes in both C₄ and CAM plants (Chollet et al., 1996), and phosphorylation of the enzyme in C₄ plants has also been shown to occur (Li et al., 1996). Both phosphorylated and dephosphorylated forms of the enzyme have been purified from C₄ plants (McNaughton et al., 1989; Arrio-Dupont et al., 1992).

The regulatory process as currently perceived to occur in C₄ plants has been reviewed in detail (Chollet et al., 1996; Gadal et al., 1996) and is outlined in Fig. 3. The dark form of the enzyme is activated by phosphorylation of a specific serine residue (residue 15 in the maize polypeptide, corresponding to residue 8 in the sorghum sequence) catalysed by a protein kinase, designated PEPC-PK. This serine residue is located in a conserved sequence motif present in all C₄ and CAM PEPC enzymes, and possibly all plant PEPCs, but absent from the bacterial enzymes, suggesting that it may have been acquired in evolution as a regulatory element. The phosphorylated enzyme exhibits greater activity at sub-saturating PEP concentration, lower sensitivity to malate inhibition, and greater activation by G6P. On illumination, phosphorylation and an increase of PEPC-PK activity occur in parallel, indicating that the protein kinase itself is subject to light regulation and that PEPC phosphorylation is subject to a regulatory cascade (Chollet et al., 1996). In darkened leaves PEPC is specifically dephosphorylated. The protein phosphatase involved is apparently active in the leaves during light and dark periods, and has been shown from inhibitor studies to resemble a mammalian type 2A protein phosphatase. Therefore, the light-dark phosphorylation status of PEPC is dependent on the mechanism and extent to which PEPC-PK activity is regulated. The focus of ongoing research is to understand the signal transduction pathway by which light-dark signals are related to enzyme phosphorylation by the PEPC-PK.

A number of protein kinase activities in plants, in addition to a number of mammalian protein kinases, are capable of phosphorylating PEPC, but only one or two are responsive to light in maize. Using a functional assay, a light-responsive PEPC-PK has been purified from maize and its properties characterized (Wang and Chollet, 1993). The native protein is a monomer of 30 kD which is not responsive to Ca²⁺ or chelating agents indicating that it is not a Ca²⁺/calmodulin-dependent type protein kinase. Later studies identified, in addition, a 37 kD protein kinase with similar properties (Li and Chollet, 1993). Whether these are separate enzymes or derivatives of the same protein has not been resolved.

The increase in PEPC-PK activity and maximum PEPC phosphorylation takes approximately 60 to 100 min to occur when darkened maize leaves are transferred to light, and dephosphorylation on darkening takes about the same time. A threshold light intensity of about 300 μmol m⁻² s⁻¹ is required in maize with little response to changes in intensity above 500 μmol m⁻² s⁻¹ (Nimmo et al., 1987), suggesting that this mechanism of regulation could be responsible for dark-light effects under normal diurnal conditions. Since the increase in PEPC-PK activity requires light and is prevented in the presence of photosynthetic inhibitors of either electron transport (Diuron or methyl viologen) or of the carbon reduction cycle (D,L-glyceraldehyde), a chloroplastic signal, which has been presumed to originate in the bundle sheath cells, has been invoked. Candidates for this signal are PGA and pyruvate (Duff et al., 1996; Giglioli-Guivarc’h et al., 1996). As the increase in PEPC-PK activity is also inhibited by cytosolic protein synthesis inhibitors, such as cycloheximide, protein synthesis is also involved. Evidence is also accumulating for a role of cytosolic pH, which becomes more alkaline on illumination (Giglioli-Guivarc’h et al., 1996). Recently, an effect of Ca²⁺ transport antagonists has been observed, leading to the suggestion that a Ca²⁺/calmodulin responsive
element may be involved (Duff et al., 1996). This has been proposed to act higher up the signal transduction pathway since, as noted above, the present view is that PEPC-PK is not Ca^{2+}/calmodulin-dependent (Chollet et al., 1996).

Since these responses to changing light conditions are relatively slow, this phosphorylation-based mechanism is apparently not involved in the regulation of PEPC during short-term fluctuations of light intensity during the day. These responses are presumably brought about by changes in substrate and effector concentrations. Regulation of PEPC is considered necessary to coordinate the C_4 and C_3 cycles, to allow PEPC to operate in the high malate concentration prevailing in mesophyll cells during photosynthesis, and, in the dark, to prevent depletion of PEP arising from glycolysis. The light-dark phosphorylation mechanism may be most important for the latter two processes, with the sensitivity to malate inhibition high in darkened leaves but decreased in the light to allow relatively high PEPC activity in the presence of high malate concentration.

A related process is thought to occur with the enzyme in CAM plants (Chollet et al., 1996). Also, as phosphorylation has been shown to occur in C_4 plants, the regulatory process may not be entirely specific to C_4 and CAM plants, although the regulatory signal transduction in C_3 species shows some differences (Li et al., 1996). It has been pointed out that the lower activity dephosphorylated form of PEPC could not support observed rates of photosynthesis at the concentrations of malate and PEP present in the mesophyll cytosol but that light induced phosphorylation and associated changes in metabolite control act synergistically to increase PEPC activity (Doncaster and Leegood, 1987; Gao and Woo, 1996a). Indeed, in contrast to C_4 plants, photosynthetic carbon dioxide assimilation was inhibited in C_4 plants when the phosphorylation state of PEPC, and the activity of PEPC-PK were decreased by short-term treatment with cytosolic protein synthesis inhibitors (Bakrim et al., 1993). This led to the suggestion that PEPC phosphorylation may be a cardinal event in regulation of C_4 photosynthesis.

B. Pyruvate, P_i, Dikinase

PPDK was discovered during a search for an enzyme to account for the conversion of pyruvate to PEP in C_4 plants (Hatch and Slack, 1968). This enzyme catalyses a unique type of reaction in which two substrates are phosphorylated using the \( \gamma \) and \( \beta \) phosphates of ATP:

\[
\text{Pyruvate} + P_i + \text{ATP} \leftrightarrow \text{PEP} + \text{PPi} + \text{AMP}
\]

Even with various precautions to maximize recovered activity, it has been difficult to demonstrate PPDK activities in C_4 leaf extracts much above the maximum photosynthesis rates for those leaves (Hatch, 1987). This, combined with the fact that the enzyme is subject to a complex mechanism of dark/light-based regulation, has led to the view that the PPDK reaction is a key site for regulation of C_4 photosynthesis. In this section we will consider the specific details of the regulation of this reaction.

Maize PPDK is a tetramer with subunit molecular weight of about 94 kDa (Sugiyama, 1973). Kinetic features are reviewed elsewhere (Ashton et al., 1990). Free Mg^{2+} is essential for activity in addition to Mg^{2+}-ATP, and both the forward and reverse rates are stimulated several-fold by the monovalent cations NH_4^+ or K^+ (Jenkins and Hatch, 1985). The regulatory significance of these cation effects is uncertain. Also of possible regulatory significance is the fact that PPDK is reversibly inactivated below 10 °C, an effect prevented by PEP or pyruvate, and is inhibited by all three products of the reaction in the direction of PEP synthesis (Edwards et al., 1985; Jenkins and Hatch, 1985). The level of the enzyme in leaves also changes in response to changing light conditions during growth (Hatch, 1978). However, the most significant regulatory process operating on PPDK is the remarkably complex and rapid dark/light-mediated modulation of its activity (Burnell and Hatch, 1985a).

Through this mechanism the enzyme is almost totally inactivated in darkened leaves, is partially activated in low light, and is completely activated after a few minutes illumination with high light. The down-regulation of PPDK in the dark presumably serves to prevent the unproductive ATP-dependent conversion of pyruvate to PEP. The same mechanism is apparently responsible for modulating PPDK activity in response to varying light in the range of up to half of full sunlight (Hatch 1981; Usuda et al., 1984). This would serve to maintain the supply of PEP in step with the prevailing light and associated demands for CO_2 assimilation and could have a rate limiting function (Usuda et al., 1984, see Section IV.C).

The processes responsible for this dark/light regulation have been described in detail (Burnell and Hatch, 1985a; Edwards et al., 1985) and the key
elements are summarized in Fig. 4. PPDK is active when a Thr residue, near a catalytic site His, is unphosphorylated. Inactivation results from the phosphorylation of this Thr but there are two remarkable features of this reaction. Firstly, ADP is the donor of the phosphate group in a reaction essentially without precedent in biological chemistry. Secondly, an absolute prerequisite for this ADP-mediated phosphorylation is that PPDK is already phosphorylated on the catalytic site His residue. In the normal course of catalysis this can occur by reacting with either ATP or PEP, thus explaining the requirement for both ADP and ATP for inactivation. Notably, pyruvate prevents inactivation by causing the removal of the catalytic site phosphate. Reactivation occurs by another unusual reaction, unique at least in terms of protein dephosphorylation. In this reaction the phosphate is removed from the regulatory Thr residue by phosphorolytic cleavage to yield inorganic pyrophosphate. Activation is much more rapid with the inactive enzyme free of the histidine phosphate and the reaction is inhibited by AMP, ADP and PPI (Fig. 4). Final confirmation that this mechanism is the basis of the dark/light regulation in leaves was provided by the studies of Budde et al., (1985) showing that the regulatory threonine is phosphorylated with PPDK isolated from darkened leaves and is removed when leaves are illuminated. The maize PPDK gene has recently been expressed in E. coli opening the way for a site-directed analysis of the function of specific catalytic and regulatory residues (Chastain et al., 1996).

To add to the unique features of this dark/light mediated regulation of PPDK, these mechanistically different activation and inactivation reactions are catalysed by the same protein entity, termed PPDK regulatory protein. Such bifunctional activity is rare and its significance for the coordinated control of PPDK activity is uncertain, although some possibilities have been discussed (Burnell and Hatch, 1986). PPDK regulatory protein has been partially purified and some of its properties described (Burnell and Hatch, 1985b; Smith et al., 1994). There are some unresolved differences with regard to the purification of this protein but agreement that it is a most difficult enzyme to work with, has a monomeric molecular weight of about 45 kDa, and can exist as a dimer or tetramer. Chloroplast ADP levels may have a critical role in regulating PPDK activity since ADP is a strong inhibitor of the phosphorolytic activation, reaction as well as being the phosphate donor for inactivation (Fig. 4; Burnell and Hatch, 1985a). PPi also inhibits activation. Smith et al., (1994) could find no evidence for regulation of the PPDK regulatory protein by either covalent modification or a mechanism based on changes of chloroplast stromal pH with changing light intensity.

The precise mechanism by which changes in irradiance elicit changes in PPDK activity through the reactions shown in Fig. 4 remains uncertain. However, possible explanations have been advanced based on changes in adenylates, P and adenylate energy charge (Edwards et al., 1985; Budde et al., 1986; Nakamoto and Edwards, 1987; Roeske and Chollet, 1989). Pyruvate levels are also likely to exert a critical regulatory influence by maintaining the active enzyme in a non-inactivatable form (see Fig. 4, Budde et al., 1986: Burnell et al., 1986). The rates of both activation and inactivation of PPDK in leaves are dramatically reduced as temperature is reduced in the range from 30 to 10 °C (Edwards et al., 1985). This is one possible explanation for the cold sensitivity of C₄ plants.

C. NADP Malate Dehydrogenase

NADP-MDH has a critical role in NADP-ME-type C₄ plants where it catalyses the reduction of OAA to malate in mesophyll chloroplasts using light-generated NADPH (Fig. 2).
This enzyme was first discovered in leaves of NADP-ME-type C₄ species where its activity is 10 to 20 times that found in other C₄ plants or in C₃ species (see Hatch, 1987). Earlier studies on the kinetic properties and regulation of NADP-MDH have been reviewed (Edwards et al., 1985; Ashton et al., 1990). NADP-MDH activity in the direction of OAA reduction is strongly inhibited by the product NADP providing simple feedback control (Ashton and Hatch, 1983). However, as discussed below, this effect may also interact synergistically with the dark/light-mediated regulation of NADP-MDH resulting from varying the NADP to NADPH ratio.

NADP-MDH is rapidly inactivated when leaves or isolated chloroplasts are darkened and is reactivated with a half time of a few minutes following subsequent illumination (Johnson and Hatch, 1970; Hatch, 1977). Activity also varies with varying light intensity in the range up to about 40% of full sunlight (Johnson and Hatch, 1970; Usuda et al., 1984). This modulation with varying light presumably serves to co-ordinate the supply of malate with the prevailing potential for CO₂ assimilation. The total inactivation of the enzyme in the dark would prevent the futile conversion of OAA to malate with associated consumption of available NADPH. There is now strong evidence that inactivation of NADP-MDH results from oxidation of dithiol groups on the enzyme forming a disulfide bond and that activation is due to the reversal of this reaction (Fig. 5). Earlier evidence for this view, and for this being a thioredoxin-m mediated reaction linked in turn to the redox state of the photosynthetic electron transport chain through ferredoxin, has been reviewed (Edwards et al., 1985).

Figure 5 outlines the mechanism of dark/light regulation of NADP-MDH as currently perceived. Sequence analysis has confirmed that the maize and sorghum enzymes contain a total of eight cysteine residues per subunit (Metzler et al., 1989; Issakidis et al., 1992). Evidence that dark/light regulation of the enzyme involves the oxidation-reduction of two pairs of these eight cysteine thiols (Jenkins et al., 1986; Hatch and Agostino, 1992) has been confirmed by site-directed mutagenesis studies (Issakidis et al., 1992, 1994). These latter studies provided evidence for which of the eight thiols are involved in the regulation of the enzyme; these are thiol pairs in short amino acid sequences situated at the N- and C-termini of the molecule that are unique to NADP-MDH. It seems likely that in the course of evolving from the NAD malate dehydrogenase gene these sequences were acquired specifically for regulation of activity. These thiol pairs apparently have different redox potentials, the one with the higher potential being readily reduced (higher redox potential, indicated by shading) but both must be reduced to give the active form of the enzyme.

The equilibrium between active and inactive forms of NADP-MDH is under complex regulation determined by the ratio of NADPH to NADP. This ratio reflects the level of light interception, the consequent redox state of the components of non-cyclic electron flow, and the demand for NADPH. This control by the NADPH to NADP ratio depends on the extreme sensitivity of the thioredoxin-mediated activation of NADP-MDH to inhibition by NADP and the reversal of this effect by higher concentrations of NADPH (Ashton and Hatch, 1983). Similar inhibition by NADP has been shown for the activation of NADP-MDH from C₄ leaves (Scheibe and Jacquot, 1983). As a result, substantial activation of the enzyme only occurs under conditions where high ratios of NADPH to NADP prevail. From a quantitative model of this system it was predicted that a ratio of about 10 would be required to give about 25% of full activity, with steeply increasing activity as the ratio increases above 10. Later experiments confirmed these predictions (Rebeille and Hatch, 1986a). Inactivation of NADP-MDH through oxidation of thiols is also inhibited by NADP but the effect is much less since the Kd for NADP binding is much higher and inhibition is more readily reversed by NADPH (Ashton and Hatch, 1983). Another critical factor
influencing the balance between the active and inactive forms of NADP-MDH is the O$_2$ concentration (Edwards et al., 1985), primarily through the oxidation of reduced thioredoxin-

Several studies with leaves and isolated chloroplasts provide support for the mechanism outlined in Fig. 5, including the critical regulatory role of the NADPH to NADP ratio (Edwards et al., 1985). In leaves, the level of NADP-MDH activation increased under conditions favoring higher electron transport redox potential and NADPH/NADP ratio, for instance, higher light, low CO$_2$ and reduced O$_2$ levels. Likewise, with isolated mesophyll chloroplasts, treatments that would decrease the electron transport redox potential or NADPH/NADP ratio decreased the level of activation of NADP-MDH. For instance, the high NADP-MDH activities recorded with illuminated chloroplasts were very substantially reduced by the inclusion of DCMU to prevent reduction of electron transport intermediates or by adding reducible substrates such as PGA or OAA which oxidize NADPH (Hatch, 1977; Leegood and Walker, 1983; Rebeille and Hatch, 1986b).

**D. NADP-Malic Enzyme**

NADP-ME catalyses the oxidative decarboxylation of malate:

\[ \text{Malate} + \text{NADP} \leftrightarrow \text{Pyruvate} + \text{CO}_2 + \text{NADPH} \]

The enzyme is widely distributed in plants and animals and it appears that, like other C$_4$ enzymes, the C$_4$ NADP-ME evolved from an existing C$_3$ form (see Marshall et al., 1996). The C$_4$ isoform, exemplified by detailed studies on enzymes from maize, sugarcane, and Flaveria species (all of the NADP-ME subgroup of C$_4$ plants), occurs at higher activities and shows some unique properties compared with other plant NADP-MEs. Most importantly, the enzyme involved in C$_4$ photosynthesis is located in the chloroplasts of bundle sheath cells, in contrast to other plant NADP-MEs which are generally cytosolic. In addition to providing CO$_2$ to the bundle sheath, the enzyme also produces reducing equivalents, particularly important in monocot C$_4$ species of this subgroup which are deficient in bundle sheath Photosystem II (see Section II.A). Extracted activities are typically two- to three-fold greater than the rate of photosynthesis in NADP-ME type C$_4$ plants, and at least 25 times higher than the activities in leaves of other subgroups of C$_4$ plants or in C$_3$ plants (Hatch, 1987). In addition to the C$_4$ isoform, which is specifically induced by light, plants of this C$_4$ subgroup also contain a ‘constitutive’ low activity cytosolic enzyme which can be measured in etiolated tissue and other non-green organs of the plant (Maurino et al., 1996). NADP-MEs of plants have been reviewed in detail (Edwards and Andreo, 1992).

Responses of NADP-ME to varying pH, substrate, and metal ion concentrations are complex and have been implicated in regulation (Edwards and Andreo, 1992). Substrate concentration responses for the C$_4$ enzymes are generally hyperbolic, few metabolites have been found to affect activity, and no allosteric properties are evident. At high malate concentration (5–10 mM) activity is maximum at pH about 8.0–8.5, which may be the pH prevailing in bundle sheath chloroplasts in the light. At lower pH, 7.0–7.5, thought to be the range prevailing in darkened chloroplasts, affinity for malate is greater but activity is very low when measured at high malate concentration due to malate inhibition, also observed with enzymes from various other sources. For the maize enzyme activity declines about 10-fold between pH 8 and pH 7. Both Mg$^2+$ and Mn$^{2+}$ can serve as cofactors with the $K_a$ for Mn$^{2+}$ generally lower. While Mg$^{2+}$ concentration is considered to increase in chloroplasts in the light, the situation for Mn$^{2+}$ is uncertain. Therefore, from these properties of the enzyme it is possible that a degree of regulatory control in response to changes in light intensity may be afforded by the stromal environment. In addition, the oligomerization state of the enzyme is somewhat dependent on pH with a more active tetramer form occurring above pH 7.5 but a less active dimer form occurring at pH 7.0; buffer type, substrates and enzyme concentrations, and the presence of reductants also influence this interconversion (Edwards and Andreo, 1992). Although isolated NADP-MEs generally do not require the presence of reduced thiol compounds for activity or to maintain stability, a possible role for thiol/disulfide interchange in regulation of the maize enzyme has been suggested (Drincoovich and Andreo, 1994).

While the maize enzyme is only inhibited weakly by the reaction products CO$_2$ and pyruvate, the enzyme mechanism indicates that NADPH should be a competitive inhibitor. Consequently, a major factor determining short-term regulation of C$_4$ NADP-MEs may be the NADP/NADPH ratio (Asami et al., 1979; Edwards and Andreo, 1992). In bundle sheath chloroplasts, malate decarboxylation by NADP-ME
is tightly coupled to PGA reduction, by NADP/NADPH cycling. Rapid control may be imposed on NADP-ME under any condition where turnover of the C₃ carbon reduction cycle is decreased. For example, on transfer from high to low light, any decrease in PGA (due to lowered ATP concentration and lower rate of RuBP carboxylation) and consequent transient increase in NADPH/NADP ratio, would decrease NADP-ME activity. This effect could be compounded by a large decline in activity if this change is accompanied by a shift of stromal pH from 8 to 7.5 or less (see above). It is possible that in the dark the poise of the NADPH/NADP ratio, combined with the lower pH, allows other biosynthetic reactions to occur but prevents malate decarboxylation.

**E. NAD-Malic Enzyme**

NAD-ME catalyses the decarboxylation of malate to produce pyruvate and CO₂:

\[
\text{Malate} + \text{NAD} \rightarrow \text{Pyruvate} + \text{NADH} + \text{CO}_2
\]

This mitochondrial enzyme is present in all higher plants and functions in respiration, allowing the tricarboxylic acid cycle to operate when glycolytic input of pyruvate is low (see Wedding, 1989). In C₄ plants, NAD-ME is located in the bundle sheath mitochondria of both NAD-ME and PCK-types and serves to decarboxylate malate transported from the mesophyll cells, providing CO₂ for Rubisco (see Fig. 2). Activities of this enzyme in NAD-ME-type C₄ leaves range from 18 to 60 times that in C₃ leaf tissue, to cope with the high flux required in photosynthesis (Hatch, 1987). In PCK-types NAD-ME primarily provides NADH for the generation of ATP for the PEPC reaction via mitochondrial electron transport (Hatch et al., 1988; Carnal et al., 1993). Many of the regulatory properties of the C₄ NAD-ME are common to the non-photosynthetic enzyme and the relevance of these to the C₄ pathway is discussed below.

Regulation of the C₄-acid decarboxylating enzymes could provide an effective control point for the coordination of the mesophyll C₄ cycle and the photosynthetic carbon reduction pathway in the bundle sheath. Since the NAD-ME reaction occurs in a non-photosynthetic compartment, i.e. the mitochondrion, some regulatory communication with other photosynthetic enzymes might be expected. Accordingly, the regulatory properties of NAD-ME have been well characterized. NAD-ME fulfills most of the criteria for a classical ‘regulatory’ enzyme (Monod et al., 1965) and is allosterically activated by a range of metabolites including acetyl-CoA, CoA and FBP (reviewed in Artus and Edwards, 1985). Although never definitively shown in C₄ plants, the C₄ and CAM enzyme can be interconverted in vitro between a low activity dimer and higher activity tetramers and octamers (see Wedding, 1989). The proportions of these polymeric forms of the enzyme are affected by the protein concentration, the ionic strength and possibly the presence or absence of activators (Wedding, 1989). Demonstrations of the relevance of this to the in vivo regulation of the enzyme are technically difficult and often unconvincing. However there is some evidence that in C₄ plants the enzyme in vivo is an octamer (Murata et al., 1989a,b).

The C₄ NAD-ME from different species falls broadly into 3 groups (see Furbank et al 1991 and references therein). The enzyme from NAD-ME-type dicotyledonous plants generally show highly sigmoidal responses to the concentration of the substrate malate and a K-type (Monod et al., 1965) allosteric activation while the enzyme from NAD-ME-type monocotyledonous plants shows simple hyperbolic kinetics and mainly V-type activation (the Vₘₐₓ increases with activation rather than the Kₘᵢₙ decreasing). NAD-ME from PCK-type species shows a mixed kinetic behavior. Potentially, in cases where extreme K-type kinetics are evident, sensitive regulation could be achieved by small changes in the levels of malate or FBP. The relevance of activation by acetyl CoA, CoA and FBP in vivo is, however, difficult to ascertain as very few studies have been made of metabolite levels in the relevant compartments of C₄ leaves. Under optimal conditions, CoA has little effect above 50 μM while mitochondrial pools could be as high as 200 μM (see Furbank et al., 1991) and FBP has not been measured in mitochondria.

Metabolites which may be of more physiological relevance in affecting NAD-ME activity are the adenylates. Furbank et al. (1991) showed that the enzyme from both NAD-ME monocots and dicots was inhibited by ATP and to a lesser degree ADP and AMP. This may be of particular relevance to the K-type enzymes where ATP at physiological levels could considerably increase the Kᵦᵦ for malate, essentially sensitizing the enzyme to falling malate levels when adenylate energy charge is low (low...
light for example). The regulation of NAD-ME in PCK-type species is interesting as, although the enzyme acts to release \( \text{CO}_2 \) in the bundle sheath, its primary role is to provide NADH to the mitochondrion for generation of ATP (see above). NAD-ME from the PCK-type species *Urochloa panicoides* is strongly activated, not inhibited, by ATP. This may provide positive feedback to \( C_4 \) acid decarboxylation by NAD-ME in the following way. When PEP-carboxykinase is operating at full capacity under conditions of high photosynthetic flux (high light for example), both ATP and OAA concentrations may increase in the bundle sheath cells. This could increase the rate of OAA entry into the mitochondria and hence its rate of reduction by NAD malate dehydrogenase with associated generation of NAD and an increase the malate pool. This, combined with ATP activation of NAD-ME could accelerate the flux of carbon through NAD-ME providing extra \( \text{CO}_2 \) (Furbank et al., 1991).

An added complication to the regulation of NAD-ME is the absolute requirement of the \( C_4 \) enzyme for \( \text{Mn}^{2+} \) (Hatch et al., 1974) and the observation that there is a strong allosteric interaction between \( \text{Mn}^{2+} \) levels and activators (Murata et al., 1989; Furbank et al., 1991). In vitro, in the absence of activator, millimolar concentrations of \( \text{Mn}^{2+} \) are required for full activity, reducing to micromolar levels in the presence of saturating CoA (Murata et al., 1989a,b). Levels of total \( \text{Mn}^{2+} \) in mitochondria are probably up to 400 \( \mu \text{M} \) but free concentrations are difficult to estimate and could be sub-micromolar (see Furbank et al., 1991) making extrapolation of the regulatory properties of this enzyme to the in vivo case very difficult.

An obvious potential regulatory mechanism for NAD-ME would be inhibition by \( \text{CO}_2 \) or bicarbonate. This would provide an effective negative feedback loop from \( \text{CO}_2 \) utilization by Rubisco. Evidence for \( \text{CO}_2 \) or \( \text{HCO}_3^- \) inhibition of NAD-ME is variable. The enzyme from *Atriplex spongiosa* and *Amaranthus edulis* is allosterically inhibited by \( \text{HCO}_3^- \) in the physiological range whereas the enzyme from * Panicum miliaceum* is largely unaffected (Chapman and Hatch, 1977). Murata et al., (1989a) found that the enzyme from *Amaranthus tricolor* showed no inhibition by \( \text{HCO}_3^- \) and inhibition by \( \text{HCO}_3^- \) of NAD-ME from the PCK-type species *Urochloa panicoides* was quite weak (Burnell, 1987). It may be that strong product inhibition by \( \text{HCO}_3^- \) is restricted to the NAD-ME-type species with a kinetically ‘\( K \)-type’ enzyme (see above) but it should also be noted that the degree of inhibition is also pH dependent (Jenkins et al., 1987).

### F. PEP-Carboxykinase

PEPCK catalyses the ATP-dependent decarboxylation of oxaloacetate producing PEP and ADP:

\[
\text{OAA} + \text{ATP} \rightarrow \text{PEP} + \text{ADP}
\]

This enzyme is the predominant decarboxylating enzyme in the PCK-type \( C_4 \) subgroup (see Fig. 2) and is located in the cytosol of bundle sheath cells. PEPCK has been purified from a number of species (Burnell, 1986) and appears to be a hexameric protein in vivo with a subunit molecular mass of about 64 kDa. Like NAD-ME, this enzyme has several kinetic properties which flag it as a possible regulatory enzyme. Interestingly, PEPCK, like NAD-ME, has a strict requirement for \( \text{Mn}^{2+} \) as a divalent metal cofactor and shows allosteric control involving this cation (Burnell, 1986). PEPCK has a binding site for \( \text{MnATP}^2- \) in addition to a separate binding site for free \( \text{Mn}^{2+} \). The binding of free \( \text{Mn}^{2+} \) influences the interaction of the enzyme with several of its metabolite effectors. PEPCK is inhibited 50–60% by PGA and FBP at concentrations around 5 mM (Hatch and Mau 1977; Burnell 1986). PGA increases the \( K_m \) of the enzyme for\( \text{MnATP}^2- \). These levels of metabolites are in the physiological range for PGA but well above measured concentrations of FBP in leaves. Dihydroxyacetone phosphate also has an inhibitory effect at physiological levels (50% at 1 mM) and appears to inhibit binding of the regulatory \( \text{Mn}^{2+} \). Bicarbonate is only a weak inhibitor of this enzyme.

Once again, because of the lack of measurements of metabolite levels in defined compartments of \( C_4 \) leaves in response to environmental changes, it is difficult to put these potential regulatory mechanisms into a physiological perspective. It is worth noting, however, that due to the low \( K_m \) of PEPCK for OAA, it has been postulated that a mechanism must exist for inactivating the enzyme in the dark (Carnal et al., 1993), otherwise cellular pools of OAA would be depleted. One hypothesis is that cytosolic \( \text{Mn}^{2+} \) levels may follow the light and dark changes in other chloroplastic cations, providing an effective light regulation of both PEPCK and NAD-ME (see Furbank et al., 1991; Carnal et al., 1993).

Recently it has been demonstrated that if rigorous
precautions are taken to prevent proteolysis after extraction, the native molecular weight of PEPC in many C₄ plants is about 68–72 kDa (Walker and Leegood 1996). PEPC is rapidly cleaved by proteolysis, removing an N-terminal extension which results in a protein of molecular weight of 62–64 kDa, the form previously reported in the literature. This higher molecular weight form is consistent with the deduced amino acid sequence of the Urochloa panicoides enzyme (Finnegan and Burnell 1995). It appears that this longer form of PEPC is phosphorylated on darkening of leaves from some but not all C₄ species (Walker and Leegood 1996; Walker et al. 1997). This covalent modification may play an important role in regulation of the enzyme in vivo, although it is not yet clear how phosphorylation affects the kinetic properties of the enzyme.

G. Enzymes of the Photosynthetic Carbon Reduction Cycle

The enzymes of the PCR cycle in C₄ plants are largely regulated in a similar manner to their C₃ counterparts (Ashton et al., 1990). For example, the three PCR cycle enzymes activated by light through the thioredoxin mediated reduction of disulfide bridges presumably play a similar role in C₄ plants, regulating fluxes and pool-sizes in the bundle sheath chloroplast. However, from metabolite pool-size measurements in maize and Amaranthus, Leegood et al., (1989) suggest that thiol mediated regulation may be less important in regulating photosynthetic flux in C₄ species. For example, FBP levels in maize do not rise as light intensity is decreased, as they do in C₃ leaves (Leegood et al., 1989), suggesting that FBPase is not down-regulated in the former case.

Worthy of special mention in the context of C₄ plants, however, is Rubisco. Regulation of this enzyme might be expected to differ from that in C₃ plants due to the high CO₂ environment of the bundle sheath cell and the unique kinetic characteristics of the C₄ Rubisco (Andrews and Lorimer 1987). It has been shown that C₄ plants contain Rubisco activase (Salvucci et al., 1987) and the tight binding ‘night-time inhibitor’ of Rubisco, carboxyarabinitol 1-phosphate (Moore et al., 1991). There are, however, very few studies on Rubisco regulation in intact C₄ leaves. In one of these studies, Sage and Seemann (1993) showed that in most C₄ species rubisco is regulated by reversible carbamylation of catalytic sites but the degree of deactivation of rubisco seen at low light varied considerably between species.

IV. Integrated Regulation of C₄ Photosynthesis

A. Rate Limiting Enzymes in the C₄ Pathway

Because of the complexity of the C₄ photosynthetic pathway it has been difficult to determine which enzymes control photosynthetic flux under a particular set of environmental conditions. In C₃ plants, the relative pool sizes of PGA, triose phosphates and RuBP can be easily measured in leaves in response to CO₂ and light and there is a detailed knowledge of the kinetics and regulation of Rubisco. In addition, several laboratories have generated transgenic plants with reduced levels of key enzymes in photosynthesis and applied control theory to assess the role these enzymes play in determining photosynthetic rate (see Furbank and Taylor, 1995 for a review). Using these measurements and models of photosynthesis it has been determined that in C₃ plants Rubisco is the major determinant of photosynthetic flux in air, at least in high light. In C₄ plants, Rubisco is not in direct communication with the atmosphere and less is known about its regulation. In addition, pools of metabolites are partitioned between organelles of the mesophyll and bundle sheath cells and there is the added complexity of the possible control of flux by the mesophyll localized enzymes of the C₄ cycle.

Until recently, the contribution of individual enzymes of the C₄ pathway to the control of photosynthetic flux has been estimated by correlating extracted enzyme activities with photosynthesis rates in comparisons between species or genotypes or within a species under different growth conditions (Furbank et al., 1997a). Experiments of this kind have provided evidence that Rubisco, PEPC, PPDK, NADP-MDH, NADP-ME and NAD-ME are all positively correlated with photosynthetic capacity (Furbank et al., 1997a). These results do not, however, provide definitive evidence that any of these enzymes limit or co-limit photosynthetic flux. Many other parameters may co-vary in these experiments and a strong positive correlation with photosynthetic performance and enzyme activity does not prove a causal relationship.

More recently, with the advent of efficient genetic transformation of the NADP-ME-type C₄ dicot
Flaveria bidentis (Chitty et al., 1994), it has been possible to apply antisense RNA technology to address this question more precisely by altering levels of a single photosynthetic enzyme in the C₄ pathway. As discussed above, antisense RNA technology has proven very useful in combination with metabolic control theory, in determining the role of an enzyme in controlling flux through metabolic pathways in plants (recently reviewed in Furbank and Taylor 1995; Stitt 1995). Figure 6A shows the expected response of flux through a pathway when the activity of a single enzyme is decreased or increased. In the case of a classical ‘rate limiting’ enzyme, flux is linearly related to enzyme level, at least up to and slightly above wild-type activities. If the enzyme is ‘non-limiting’, transformants with greatly reduced levels of the enzyme will still support the same flux as wild-type individuals. In reality, enzymes tend to be ‘co-limiting’ (i.e. a curvilinear relationship between enzyme level and flux). In the case of co-limiting enzymes, their contribution to the control of flux is given by the tangent to the curve of flux versus enzyme activity at close to wild-type enzyme levels. This slope is the ‘control coefficient,’ Cᵢ (Kacser and Burns 1973). An enzyme with Cᵢ = 1 has 100% control over flux while a Cᵢ of zero indicates no control. Using the Flaveria transformation system, transgenic plants have been produced with reduced levels of Rubisco (Fig. 6B; Furbank et al., 1996), PPDF (Fig. 6C; Furbank et al., 1997a) and NADP-malate dehydrogenase (Fig. 6D; Trevanion et al., 1997). Using this analysis, we determined that under high light and atmospheric CO₂ levels, Cᵢ for Rubisco was 0.6 (or greater) while for PPDF, Cᵢ was between 0.2 and 0.4 and for NADP-MDH, Cᵢ was zero (Furbank et al., 1997a). This control strength for Rubisco is similar to that determined for the C₄ plant tobacco under saturating illumination (Furbank and Taylor, 1995). As light intensity was decreased, the control of photosynthesis by both PPDF and Rubisco quickly fell to zero as the provision of light generated ATP and reductant presumably became limiting. These results indicate that at high light intensities, control of photosynthetic flux in C₄ plants is predominantly shared between Rubisco and PPDF with the bulk of control residing with the former enzyme. Recent evidence from mutants of PEPC and NAD-ME in Amaranthus suggests that the control
strength of PEPC may also be significant and of the same magnitude as PPDK under high light conditions (Dever et al., 1995, 1997).

It is interesting to note that NADP-MDH levels in Flaveria leaves are far in excess of those required for photosynthesis even when activation state in vivo is taken into account (Trevanion et al., 1997; Furbank et al., 1997a). Despite a complex covalent regulation mechanism which causes NADP-MDH activity to track photosynthetic rate, at no time is it ‘rate limiting’ (Furbank et al., 1997a). This observation shows the danger in using correlative evidence on enzyme levels and photosynthetic rate in wild-type plants to determine the role of an enzyme in controlling flux. It also poses the question of the necessity for regulation of these ‘non-limiting’ enzymes. We recently suggested (Furbank et al., 1997a) that conservation of metabolite levels may be the reason such complex regulation has evolved. For instance, the Km of NADP-MDH for NADPH is quite low (around 50 μM) and without redox regulation of NADP-MDH and the complex regulation of activity by NADPH/NADP ratio (see Section III.C. and Fig. 5), chloroplast NADPH pools would be depleted to very low levels and the NADPH/NADP ratio would decline to around 0.05 (calculated in Furbank et al., 1997a). Such low levels of NADPH would effectively starve other biosynthetic processes and could cause instability during transients such as light-flecks, for example. A similar regulatory process has previously been proposed in C3 plants for the enzymes of the PCR cycle (Woodrow et al., 1985) and dark inactivation of other high activity, low Km enzymes of the C4 pathway may be necessary (see above).

B. Coordination of the C3 and C4 Cycles

Although the mesophyll ‘CO2 pump’ cycle essentially operates independently of the PCR cycle in the bundle sheath, there are a number of biochemical links between the two cell types which could provide co-ordinate regulation of the two cycles. The most basic level of coordination is provided by the relative amounts of PEPC and Rubisco present in the leaf. These ratios can vary considerably between species (von Caemmerer and Furbank, 1999) but as the maximum extractable activity of PEPC in most C4 leaves is far above the photosynthetic flux, the significance of this observation is uncertain. Changes in the levels of C4 enzymes in response to environmental conditions will be dealt with separately, but one would expect the co-ordinate regulation of expression of photosynthetic enzymes to be under strong genetic control (see Furbank and Taylor 1995). The following discussion will be confined to potential co-ordinate enzyme regulation and communication between the cell-types due to the levels and movement of metabolites.

Evidence for fine co-ordinate control of mesophyll and bundle sheath reactions is rare. Recent work using transgenic Flaveria with reduced levels of Rubisco (Furbank et al., 1996; von Caemmerer et al., 1997) suggests that unless Rubisco is reduced to quite low levels, no large-scale co-ordinate down-regulation of PEP carboxylation occurs. This is evidenced by the fact that with moderate reductions in the level of Rubisco, the initial slope of the A versus Ci response curve, determined by the kinetics and amount of PEPC, is unaffected (von Caemmerer et al., 1997). In these low Rubisco plants, if no down-regulation of the mesophyll reactions were to occur, CO2 should build up in the bundle sheath compartment. This in fact does happen but to a lesser degree than that predicted by modeling of carbon isotope discrimination and photosynthesis (von Caemmerer et al., 1997), suggesting that some down-regulation of flux through the C4 cycle does occur in the transformants. On balance, it appears that some regulatory coupling of the C3 and C4 cycles is occurring in these plants but the coupling is by no means absolute.

One way in which the C3 and C4 cycles could be in regulatory communication is through the traffic of metabolites and in particular through the interconversion of PEP and the 3-carbon sugar phosphates. In this case, it has been shown that during photosynthetic induction in maize leaves, large pools of triose phosphate are built up by carbon draining from the C4 acid pool to PEP and then to PGA via phosphoglycerate mutase and enolase (Leegood and Furbank, 1984; Furbank and Leegood, 1984). There is also evidence for this pathway operating in sugarcane from randomization of label in the early 14C labeling experiments of Hatch and Slack (1966) and from metabolite measurements in leaves of Amaranthus edulis at varying CO2 concentrations and irradiances (Leegood and von Caemmerer, 1988). This mechanism may be particularly important in NADP-ME type monocots. In many of these species there is little or no Photosystem II in the bundle sheath chloroplasts (Hatch, 1987), necessitating a
large traffic of PGA to the mesophyll chloroplasts for reduction. If this pool were to exchange freely with the mesophyll PEP pool, flux through the two pathways could be co-ordinated. In addition, this traffic of PGA and triose phosphate between the cell types could modulate activity of PEPC as triose phosphate (and hexose phosphates produced during sucrose synthesis in the mesophyll) are potent activators of this enzyme (Leegood et al., 1989). Additional coordination could operate at the level of reducing power in NADP-ME-type species because NADP-ME in the bundle sheath chloroplast generates part of the NADPH used in the PCR cycle (Fig. 2 and Hatch, 1987).

Another possible regulatory link between the C₃ and C₄ cycles is through regulation of C₄ acid decarboxylation. This could occur at two levels: firstly, by direct modulation of enzyme activity, largely dealt with in section III, and, secondly, through control of metabolite transport into the bundle sheath organelles. In the NAD-ME-type and NADP-ME-type species where C₄ acid decarboxylation occurs in the mitochondrion and the chloroplast, respectively, the transport of C₄ acids could be controlled by the levels of co-transported or antiported metabolites (Fig. 2 and Chapter 20, Dengler and Taylor). In the case of the NAD-ME types, this may be of particular relevance through the transport of aspartate and 2-oxoglutarate into the mitochondrion (Hatch, 1987). In experiments with both isolated mitochondria and bundle sheath cells from these species, malate and Pi must be added together with aspartate and 2-oxoglutarate to support high rates of pyruvate production (Furbank et al., 1990a). It has been proposed that the Pi and malate requirements are due to the counter exchange of 2-oxoglutarate and malate which in turn depends upon a Pi/malate antiporter (Furbank et al., 1990a). Although there is no net consumption of malate, this mechanism would provide a sensitive regulatory mechanism responsive both to cytosolic Pi (allowing positive feedback from sucrose biosynthesis) and malate levels. Malate is a significant component of the C₄ acid pool formed during photosynthesis in NAD-ME types (although aspartate is the predominant C₄ acid transported in this type) and the ratio of malate to aspartate formed would be responsive to chloroplast redox state through the activity of mesophyll NADP-MDH. Since malate levels in the bundle sheath would be in equilibrium with those in the mesophyll, malate level could provide both a feed-back and feed-forward regulatory mechanism, indirectly responsive to light.

In summary, there is circumstantial evidence for coordinate regulation of the C₃ and C₄ cycles in vivo and, intuitively, one would expect some regulation of the flux in the two compartments. However, we are still some way from understanding how these processes operate in an intact leaf.

C. Regulation in Response to Light Intensity

A useful approach to understanding integrated regulation of C₄ photosynthesis has been to examine the response of photosynthetic rate, enzyme activity and metabolite levels to changes in light intensity (reviewed by Leegood et al., 1989). Using these techniques to examine the response of photosynthesis in maize to a step change in irradiance from 1700 to 140 μmol m⁻² s⁻¹, Leegood et al. (1989) concluded that flux immediately following this transition was predominantly determined by ATP supply, not enzyme regulation. For example, they observed a rapid build up in pyruvate but only a slow reduction in PPDK activity to about 50% of high light values. Triose phosphate levels rapidly declined in low light, coincident with a fall in thylakoid energization, also supporting the hypothesis that ATP became limiting.

An examination of leaf metabolite levels at steady-state over the light response curve in C₄ plants in air shows a strong correlation between triose phosphate pools and photosynthetic flux (Leegood et al., 1989 and references therein). PEP levels on the other hand remain remarkably constant, prompting the authors to conclude that PEPC activity may be regulated to match photosynthetic flux through changes in levels of the activator triose phosphate.

Co-ordinate regulation of electron transport and carbon metabolism in C₄ plants appears to follow much the same pattern as in C₃ photosynthesis. Photosynthetic control of electron transport has been shown to operate in isolated maize mesophyll chloroplasts both at the level of proton back-pressure, reducing the rate of inter-system electron transfer and to a lesser degree via negative feedback on the quantum efficiency of Photosystem II, resulting from a high thylakoid ΔpH (Furbank 1988, Foyer et al., 1990). As quite high light intensities are required to saturate photosynthesis in C₄ plants, it is likely that regulation of photosynthesis by ATP supply and the interplay between electron transport and carbon metabolism could be of great importance in C₄ plants over a wide range of normal growth irradiances.
D. Adaptive Responses to Environmental Changes

Regulation of C_4 photosynthesis in terms of its longer term adaptive response to environmental variables such as light and temperature, as well as major nutrients such as nitrogen and phosphate will be considered briefly here. It is worth noting that when plants are exposed to suboptimal light, temperature, or nutrient levels during their whole growth period, photosynthesis is almost invariably reduced. However, many components of photosynthesis can be affected under such conditions and in C_4 plants effects are not generally specific to the C_4 processes. In such situations it is very difficult to dissect out any effects of the altered environmental parameter on C_4 processes specifically. A potentially more useful approach to assess these effects has been to examine changes in photosynthesis and the C_4 photosynthetic components in the short term (within the range of minutes to days) in response to an environmental change, after growth under an alternative condition. Relatively fewer such studies have been carried out.

An exception to this is the response of C_4 photosynthesis to low temperature, which has been extensively examined. In general, C_4 plants respond poorly to low temperature and photosynthetic efficiency becomes lower than that of C_3 species as temperature decreases (see Section II.B). The day and night minimum temperature during growth appears to be a major determinant of the distribution of C_4 plants, both in their natural ecosystems and in agricultural situations (Chapter 22, Rawsthorne and Monson). Because of this it has been tempting to consider that the poor growth at low temperature is closely related to C_4 physiology or biochemistry (reviewed by Long, 1983). However, despite many correlative studies of the low temperature sensitivity of a number of C_4 processes, no single causative factor or mechanism has clearly emerged. Physiological processes suggested to be involved in this low temperature sensitivity of C_4 plants have included the sensitivity of specific enzymes to low temperature, particularly PPDK and PEPC (Long, 1983; Usami et al., 1995; Krall and Edwards, 1993), translocation processes (Long, 1983), and, in earlier studies, carbohydrate loading in the bundle sheath chloroplasts leading to inhibition of photosynthesis (summarized by Ku et al., 1978). PEPC, as well as PEPC has been implicated in a recent comparison of cold-sensitive and tolerant PCK-type species (Matsuba et al., 1997). Whether there may be a more subtle indirect effect of low temperature on a C_4-specific process, for example, regulation of gene expression, or an enzyme regulatory mechanism has not yet been explored adequately. In relation to this, specific effects of low temperature on activation of C_4 enzymes, particularly PPDK, were observed in maize and sorghum (Taylor et al., 1974) and, recently, in Echinochloa crus-galli populations (Simon and Hatch, 1994). The low temperature effects on C_4 photosynthesis and growth of C_4 plants have been reviewed by several authors (Berry and Bjorkman, 1980; Miedema, 1982; Long, 1983).

Regarding regulation of C_4 photosynthesis in response to varying light intensity, there will be changes in the short term which presumably involve coordination between the C_4 and C_3 cycles, as discussed earlier in this section. The possible influence of light intensity in the regulation of activity of particular C_4 cycle enzymes, particularly PEPC, PPDK, and NADP-MDH, has been covered in Section III. In addition to these effects, adaptive changes of C_4 cycle enzyme activities have been shown to occur over several days, in response to changes in light intensity at which the plants are growing. In mature leaves, transfer from low to high light was accompanied by increases in PEPC and PPDK and on transfer from high to low light corresponding decreases occurred (Hatch et al., 1969). Interestingly, changes in RuBP carboxylase and other C_3 cycle enzymes were less marked. Later studies of acclimation during growth under various light intensities have also indicated a degree of regulation of the levels of C_4 enzymes (Usuda et al., 1985; Ward and Woolhouse, 1986). It is not established whether this control of enzyme level in mature leaves is primarily at the level of transcription or protein turnover. However, during greening of etiolated leaves, or in early leaf development, control of enzyme synthesis for most, if not all, C_4 enzymes appears to be transcriptional (Chapter 21, Pearcy and Sage). While irradiance itself may be the primary signaling factor controlling the level of C_4 cycle enzymes, expression of the genes for PEPC, PPDK, carbonic anhydrase and Rubisco are also affected by nutrient status and developmental cues, indicating that regulation is likely to be complex. In a C_4 Flaveria species, enzyme level appeared to respond to supplied carbohydrate rather than light directly (Furbank et al., 1997b).

The effects of changes in the supplied levels of the
nutrients, inorganic nitrogen and phosphate, on photosynthesis and enzymes in C₄ plants have also been examined. Phosphate deficiency dramatically decreased photosynthesis in maize, with most photosynthetic enzymes declining, including PPDK, PEPC and many C₃ cycle enzymes, in contrast to other cytosolic enzymes such as sucrose-phosphate synthase and UDP-glucose pyrophosphorylase (Usuda and Shimogawara, 1992). PEPC, PPDK and carbonic anhydrase protein levels also respond to increasing nitrate, with control at the transcriptional level since mRNA also increases (Sugiyama et al., 1984; Yamazaki et al., 1986; Burnell et al., 1990; Sugiharto et al., 1992). As in C₃ plants, photosynthetic capacity of C₄ plants is clearly related to the level of N-supply during growth (Wong, 1979).

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Summary

C₄ photosynthesis involves intercellular metabolite transport, between the mesophyll and bundle-sheath, and intracellular shuttling of metabolites between the chloroplasts, cytosol and, in some cases, the mitochondria. In this chapter, the role of diffusion driven intercellular transport of metabolites via plasmodesmata is discussed, together with the impact on the ability of the bundle-sheath to concentrate CO₂. The chapter also considers the nature and role of organelle transporters for phosphorylated compounds, organic and amino acids in relation to C₄ photosynthesis in the mesophyll and bundle-sheath.

I. Introduction

Photosynthetic CO₂ fixation in C₄ plants depends crucially on metabolite transport, not only within cells, but also between mesophyll and bundle-sheath cells (hence C₄ photosynthesis has been termed ‘co-operative photosynthesis’ by Karpilov (1970)). This transport must occur at high rates which are equivalent to rates of photosynthesis. The operation of C₄ photosynthesis has far-reaching consequences for the structure of the leaves of C₄ plants. The necessity for metabolite transport between the mesophyll and bundle-sheath requires intimate contact between the cells and therefore limits the amount of mesophyll tissue which can be functionally associated with bundle-sheath tissue. The leaf thickness is limited in C₄ plants and the interveinal distance (i.e. the number of mesophyll cells between adjacent bundle-sheaths) is characteristically smaller than in the leaves of C₃ plants, about 300 μm for C₃, and 100 μm for C₄, grasses (Kawamitsu et al., 1985), although there are appreciable differences in interveinal distance between the various C₄ decarboxylation types (Dengler and Nelson, 1999). Mesophyll cells are never more than one cell removed from bundle-sheath cells (Hattersley and Watson, 1975).

The intracellular metabolite transport between organelles is as important to the operation of C₄...
photosynthesis as intercellular metabolite exchange and it must also proceed at comparable fluxes. In the mesophyll, metabolite exchange occurs between chloroplasts and cytosol, while in the bundle-sheath, the mitochondria are also involved in the photosynthetic metabolism of NAD-ME and PCX-types (Chapter 18, Furbank et al.). In NAD-ME plants in particular, malate decarboxylation in the mitochondria will proceed at rates several-fold higher than the high rates of glycine decarboxylation which occur during photorespiration in C₃ plants (Chapter 5, Douce and Heldt).

II. Intercellular Metabolite Transport in C₄ Plants

The most important requirement for intercellular diffusion of metabolites is the presence of plasmodesmata which permit the rapid exchange of solutes between cells (Lucas et al., 1993; Chapter 11, Schobert et al.). Although it has been suggested that active transport of metabolites occurs (Oleson 1975; Raghavendra and Das 1978), the evidence strongly supports the view that metabolites diffuse freely between the mesophyll and bundle-sheath cells and, in any case, the bundle-sheath cell wall in many C₄ plants is heavily suberized, which would restrict apoplastic movement of solutes. Extensive pit-fields with plasmodesmata on the wall between mesophyll and bundle-sheath cells provide symplastic connections between the different cells. The plasmodesmata are particularly frequent in primary pit-fields at the areas of contact between mesophyll and bundle sheath cells, and in two C₄ grasses, Themeda triandra and Panicum maximum, the majority of the plasmodesmata in vascular bundles are at this interface (56% and 77%, respectively), compared to only 32% in a C₃ grass (Bromus unioloides) (Botha and Evert, 1988; Botha, 1992). The involvement of plasmodesmata in transport of photosynthetic intermediates is also indicated by the fact that the plasmodesmal frequency is related to the CO₂ assimilation rate (Botha, 1992). The cross-sectional area of the spherinetr in plasmodesmata occupies 1.5–3% of the pit-field in the surface between mesophyll and bundle sheath cells. The plasmodesmata exclude large molecules, such as cytosolic proteins, and the size exclusion limit in isolated bundle-sheath strands is about 900 Da (Burnell, 1988; Weiner et al., 1988; Valle et al., 1989), although there may be regulation of plasmodesmal permeability in vivo (Chapter 11, Schobert et al.). Small molecules probably diffuse through micro-channels between the globular proteins in the appressed endoplasmic reticulum-protein complex (the desmotubule). The distances involved are small (1 μm or less) and intercellular metabolite exchange may be accomplished in 50 to 100 ms (Lucas et al., 1993).

Osmond (1971) used an analogy based on symplastic transport of solutes across the concentric cylinders of cortical and vascular tissue in roots and concluded that metabolite transport in C₄ photosynthesis could be sustained solely by diffusion, driven by gradients in the concentrations of metabolites. In the case of malate or aspartate, Hatch and Osmond (1976) estimated that a gradient with the concentration in mesophyll source cells 10 mM higher than in bundle sheath sink cells would be needed to sustain observed rates of photosynthesis in maize (which has centrifugally arranged bundle-sheath chloroplasts and thus a short diffusion path) and 30 mM in Amaranthus (which has centripetally arranged chloroplasts and, therefore, a longer diffusion path). Subsequent direct measurement of diffusion constants in isolated bundle-sheath strands for a range of small molecular mass compounds (values of ca. 3 μmol min⁻¹ mg⁻¹ chlorophyll mM⁻¹) has resulted in a revision of the required gradient down to 2 mM (Weiner et al., 1988). Another factor that could influence metabolite transport is the movement of water through the transpiration stream, which would aid movement from the bundle-sheath to the mesophyll and hinder movement in the opposite direction. Its magnitude, however, is likely to be less than 1% of the fluxes driven by metabolite concentration gradients (Stitt and Heldt 1985b).

Aside from the structural features which allow rapid intercellular communication, there is metabolic evidence for metabolite transport between the mesophyll and bundle-sheath. First, rapid metabolite movement may be inferred from the rapid transfer of ¹⁴C from C₄ acids (labeled in the mesophyll) to glyceraldehyde-3-P and products (which are labeled in the bundle sheath) following the supply of ¹⁴CO₂ to leaves of C₄ plants (Hatch and Osmond, 1976). Such a transfer can also be visualized by microauto-

**Abbreviations:** DHCP – dihydroxyacetone phosphate; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; PK – phosphoenolpyruvate carboxykinase; PEP – phosphoenolpyruvate; Rubisco – ribulose-1,5-bisphosphate carboxylase/ oxygenase
radiography of leaves from pulse-chase experiments. For example, after a 2 s pulse of $^{14}$CO$_2$ (when the majority of the label is in C$_4$ acids), the cytosol of the mesophyll cells is clearly labeled. However, a considerable amount of label is already found in bundle-sheath cells, and the majority of the label is transferred to the bundle-sheath during a 10 s chase (Osmond, 1971). Second, in all C$_4$ subtypes, the reduction of glyceraldehyde-3-P to triose-P is shared between the Benson-Calvin cycle in bundle sheath chloroplasts and the mesophyll chloroplasts. This requires intercellular transport of these metabolites in addition to the C$_4$ acids (malate and aspartate) and C3 acids (pyruvate, PEP and alanine) of the C$_4$ cycle. Metabolite measurements on intact leaves of maize show that the amounts of glyceraldehyde-3-P and triose-P are extremely high when compared with C$_3$ species (Leegood and Furbank 1984; Usuda 1987a,b; Leegood and von Caemmerer 1989) and amounts of triose-P are typically 20 times higher than in the leaves of C$_3$ plants. These large amounts reflect the concentration gradients of these metabolites within the leaf (Leegood and Furbank 1984; Tables 1 and 2).

Direct measurements of the gradients of metabolites in leaves of maize have been made by Leegood (1985), by Stitt and Heldt (1985a,b) and by Weiner and Heldt (1992). These are shown in Tables 1 and 2. These measured gradients are clearly higher than the predicted gradient of 2 mM (Weiner et al., 1988) and are sufficient to support metabolite transport at rates equal to the rate of photosynthesis. The most striking feature is the high contents of glyceraldehyde-3-P and triose-P and their asymmetric distribution, with the major portion of the triose-P within the mesophyll and the major part of the glyceraldehyde-3-P within the bundle-sheath, confirming the existence of metabolite gradients involving these compounds and the operation of a glyceraldehyde-3-P/triose-P shuttle between the mesophyll and bundle-sheath. Non-aqueous fractionation of maize leaves has subsequently shown that these gradients of glyceraldehyde-3-P and triose-P exist between the cytosols of the mesophyll and bundle-sheath (Table 2)(Weiner and Heldt, 1992). The fact that the apparent gradient of pyruvate lies in the opposite direction to the expected flux originally suggested that this might be the result of the intracellular accumulation of pyruvate within mesophyll chloroplasts (a feature which was subsequently demonstrated using intact chloroplasts, Flügge et al., 1985, see below). There is, therefore, almost certainly a gradient of pyruvate between the bundle-sheath and the mesophyll cytosol in vivo. Efficient regulation of metabolism is required if these metabolite gradients are not to collapse. In maize, interchange of carbon between PEP and glyceraldehyde-3-P (Furbank and Leegood, 1984) must be curtailed in the bundle sheath in order to prevent the collapse of the gradient of glyceraldehyde-3-P, as must the overall conversion of pyruvate to triose phosphate and of glyceraldehyde-3-P to malate in the mesophyll.

Considerable technical difficulties still surround the measurement of the gradient of malate, since much (99%) of the malate pool is non-photosynthetic and is present in the vacuole or in non-photosynthetic leaf cells. These large malate pools readily contaminate the cellular or sub-cellular fractions obtained from intact leaves. No direct measurements have been made of metabolite gradients in leaves of C$_4$ plants other than maize. In leaves of *Amaranthus edulis*, an NAD-ME enzyme species, aspartate is transferred from the mesophyll to the bundle sheath and alanine is returned to the mesophyll cells. It has been shown that amounts of aspartate and alanine in leaves of *A. edulis* are sufficient to account for diffusion-driven transport of these compounds between the mesophyll and bundle sheath cells under many different flux conditions (Leegood and von Caemmerer, 1988). In PCK-type plants, transport may be rather more complex, as both malate and aspartate must be transferred from the mesophyll to

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration (mM)</th>
<th>Metabolite gradient Δ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mesophyll</td>
<td>bundle-sheath</td>
</tr>
<tr>
<td>glycerate-3-P</td>
<td>5.2</td>
<td>14.7</td>
</tr>
<tr>
<td>triose-P</td>
<td>11.9</td>
<td>2.8</td>
</tr>
<tr>
<td>malate</td>
<td>56.5</td>
<td>13.3</td>
</tr>
<tr>
<td>pyruvate</td>
<td>7.4</td>
<td>6.3</td>
</tr>
<tr>
<td>CO$_2$ + HCO$_3^-$</td>
<td>0.03</td>
<td>0.6</td>
</tr>
</tbody>
</table>
the bundle sheath, and both PEP (and possibly some pyruvate) and alanine return to the mesophyll (Chapter 18, Furbank et al.; Fig. 2). Such a situation will also obtain in NADP-ME type plants which utilize PCK in the decarboxylation of aspartate, such as maize (Wingler et al., 1999). In both these C4 types, if amino acids, such as aspartate, enter the bundle-sheath and phosphorylated compounds, such as PEP, return, this would also necessitate shuttles of Pi and glutamate/2-oxoglutarate between the two cell types. Gradients of 2-oxoglutarate and glutamate are entirely feasible (Leegood, 1985; Weiner and Heldt, 1992; Table 2). In addition, inter- and intra-cellular transport will be more complex in those NADP-ME species that show mixed formation of malate and aspartate, such as Flaveria bidentis (Meister et al., 1996) and in NADP-ME species that also contain NAD-ME, such as Neostapfia colusana (Keeley, 1998).

A. Implications of Intercellular Transport for Regulation of C4 Photosynthesis

Coordination of the rate at which the Benson-Calvin cycles fix CO2 is necessary if photosynthesis is to proceed efficiently under different environmental conditions. The breakdown of such coordination during light flecks, for example, has been shown to result in inefficient CO2 assimilation (Krall and Pearcy 1993). Reduction of glycerate-3-P, deriving from the bundle-sheath, in the mesophyll occurs in all C4 species (Hatch and Osmond, 1976) and is probably a major component in this co-ordination. The triose-P which is formed from glycerate-3-P in the mesophyll can either be utilized in carbohydrate synthesis or return to the bundle-sheath chloroplast.

An example of such co-ordination is to be found in the regulation of PEP carboxylase (Fig. 1). Products of glycerate-3-P reduction in the mesophyll, such as triose-P, and ultimately hexose-P, act as positive effectors of PEP carboxylase, and relieve inhibition by malate. They can be considered as metabolite ‘messages’ from the bundle-sheath. In leaves of maize, Amaranthus edulis and Flaveria bidentis, the amount of triose-P is always closely related to the assimilation rate whether the flux is changed by alterations in irradiance or CO2 (Leegood and von Caemmerer 1988, 1989, 1994). Increasing concentrations of triose-P and hexose-P (indicating increased output from the Benson-Calvin cycle) would then increase the activity of PEP carboxylase which would increase the rate of fixation in the mesophyll. On the other hand, if too much glycerate-3-P were diverted to PEP (Furbank and Leegood, 1984) and then to malate because of an excessive activity of PEP carboxylase, this would lead to accumulation of inhibitors of PEP carboxylase if the CO2 could not be fixed at similar rates by Rubisco. The decrease in glycerate-3-P would lead to decreases in the amounts of the activators of PEP carboxylase, triose-P and aspartate, if the CO2 could not be fixed at similar rates by Rubisco. The decrease in glycerate-3-P would lead to decreases in the amounts of the activators of PEP carboxylase, triose-P and aspartate, and thus decrease the rate at which glycerate-3-P is consumed. As another example, photorespiratory intermediates, such as glycine and serine, which accumulate in leaves when the intercellular concentration of CO2 falls (Leegood and von Caemmerer 1994), can activate PEP carboxylase, particularly in dicots (Nishikido and Takanashi 1973; Doncaster and

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Content (nmol mg⁻¹ chlorophyll)</th>
<th>bundle-sheath</th>
<th>extra-chloroplast</th>
<th>mesophyll</th>
<th>chloroplast</th>
<th>extra-chloroplast</th>
</tr>
</thead>
<tbody>
<tr>
<td>malate</td>
<td>31</td>
<td>2970</td>
<td></td>
<td>78</td>
<td>821</td>
<td></td>
</tr>
<tr>
<td>glycerate-3-P</td>
<td>348</td>
<td>243</td>
<td></td>
<td>113</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>triose-P</td>
<td>45</td>
<td>29</td>
<td></td>
<td>86</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>alanine</td>
<td>896</td>
<td>928</td>
<td></td>
<td>480</td>
<td>896</td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td>60</td>
<td>80</td>
<td></td>
<td>44</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>serine</td>
<td>86</td>
<td>67</td>
<td></td>
<td>50</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>aspartate</td>
<td>308</td>
<td>220</td>
<td></td>
<td>308</td>
<td>264</td>
<td></td>
</tr>
<tr>
<td>glutamate</td>
<td>329</td>
<td>180</td>
<td></td>
<td>360</td>
<td>336</td>
<td></td>
</tr>
<tr>
<td>glutamine</td>
<td>123</td>
<td>82</td>
<td></td>
<td>110</td>
<td>95</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Contents of metabolites in non-aqueously prepared fractions of maize leaves. From Weiner and Feldt (1992). If the volume of the cytosol and chloroplasts is 20 μl mg⁻¹ chlorophyll, then the following figures divided by 20 will give an approximate mM concentration. The volume of the cytosol is 45 μl mg⁻¹ chlorophyll in Panicum miliaceum bundle-sheath cells (Valle et al., 1989).
Leegood (1988), and probably again act as metabolite messages, transmitting information about the rate of fixation in the bundle-sheath.

III. Gaseous Fluxes Between Bundle-Sheath and Mesophyll

An important feature of transport between the mesophyll and bundle-sheath is the extent to which the structural modifications which allow metabolite transport also allow leakage of out of the bundle-sheath. If appreciable leakage occurred, this would reduce the efficiency of photosynthesis. Thus a leak rate of 50% (equivalent to 100% overcycling of the acid cycle; Furbank et al., 1990a) would increase the quantum requirement for photosynthesis by about 4 mol quanta.

(Hatch et al., 1995). Modeling of the pool of indicates that the pool of in the bundle-sheath is about 70 some 15 times ambient (Jenkins et al., 1989b). Modeling of the inorganic carbon pool in mesophyll and bundle sheath cells has shown that the efflux of via plasmodesmata is insignificant compared to the flux of acids (Furbank and Hatch, 1987). Leakage of via the plasmodesmata is not appreciable, nor is the leakage of CO₂, because the diffusion coefficients of gases in solution are 10⁴ times less than in air.

Leakage of CO₂ from the bundle-sheath is likely to be influenced by the occurrence of a suberized lamella in the bundle-sheath cell wall. A suberized lamella is absent in dicotyledonous species, and in grasses is present only in species with either an uneven bundle sheath outline or with centrifugally located chloroplasts. In those species with uneven cell outlines the suberized lamella may be important in restricting CO₂ leakage through the high surface area of the bundle sheath/mesophyll interface (Hattersley, 1992). Hatcher and Osmond (1976) and Hattersley and Browning (1981) have suggested that features of the leaf anatomy of NAD-ME monocots and dicots may compensate for the lack of a suberized lamella, so that the conductances to diffusion of CO₂ from the bundle-sheath need not be different. These include the location of the chloroplasts in a centripetal position in the bundle-sheath so that the diffusion pathway for CO₂ to the mesophyll is longer, and a higher bundle-sheath surface-to-volume ratio, especially for monocots (Hattersley and Browning, 1981). However, estimates made by Jenkins et al., (1989a) and by Furbank et al., (1989) suggest that, on average, those species with a suberized lamella do have a lower physical conductance to diffusion of CO₂ than those without it.

The leakiness (the fraction of released by acid decarboxylation in the bundle-sheath which subsequently leaks out to the mesophyll) is determined not only by the physical conductance of the bundle-sheath but also by the relative capacities of the C₄ cycle to generate CO₂ and of the Benson-Calvin cycle to fix it and is thus a measure of the extent to which PEP carboxylations exceed carboxylations by Rubisco. Henderson et al., (1992) suggest that, once the bundle-sheath conductance to CO₂ is sufficiently low, leakiness may be largely determined by the relative activities of Rubisco and PEP carboxylase. Henderson et al., (1992) have employed short-term measurements of carbon isotope discrimination to estimate leakiness. For Sorghum bicolor and Amaranthus edulis, leakiness was estimated at 0.2 and was little affected by irradiance, temperature or CO₂ partial pressure, although it increased at low irradiance. For a range of monocots and dicots of various decarboxylation types, leakiness was also around 0.2, and was not correlated with the presence or absence of a suberized lamella which surrounds the bundle-sheath, but it was significantly
higher in two species: Elusine coracana (NAD-ME; 0.3) and Chloris gayana (PCK; 0.25). Hatch et al., (1995) assessed leakiness by measuring the amount of $^{14}$CO$_2$ released from leaves during a chase in cold CO$_2$. For nine species of grasses representing the different C$_4$ subgroups and two dicotyledonous NAD-ME species, the CO$_2$ leak rate ranged between 8 and 14%. However, very high CO$_2$ leak rates (averaging about 27%) were recorded for two NADP-malic enzyme type dicotyledonous species of Flaveria (F. bidentis and F. trinervia). This may be reflected in the observed low quantum yield in these Flaveria species (Monson et al., 1986). Jenkins et al., (1989a) also used a specific inhibitor of PEP carboxylase, DCDP (3,3-dichloro-2-(dihydroxy-phosphinoyl-methyl)-propenoate) to inhibit photosynthesis in leaves of various C$_4$ plants. Photosynthesis could be restored by elevating the CO$_2$ concentration to about 0.8% and above. External CO$_2$ at a sufficiently high concentration will then lead, by diffusion through the bundle-sheath cell wall, to appreciable CO$_2$ concentrations in the bundle-sheath which can be fixed directly by Rubisco. A similar phenomenon is observed in mutants of Amaranthus edulis lacking PEP carboxylase, DCDP (Dever et al., 1995). Permeabilities to CO$_2$ were approximately 100-fold lower than values for the permeabilities of mesophyll cells of C$_4$ plants (Jenkins et al., 1989a; Brown and Byrd 1993). Leakiness of the bundle-sheath to CO$_2$ was estimated at about 0.14.

Consideration of the reduced permeability of the bundle-sheath to CO$_2$ then raises questions about the O$_2$ concentration in the bundle-sheath. C$_4$ plants that do not evolve O$_2$ in the bundle-sheath because they lack photosystem II (i.e. NADP-ME species) present the simplest picture, but there have been no direct measurements of the concentration of O$_2$ in the bundle-sheath. Burnell and Hatch (1988a) have estimated that the O$_2$ concentration in the bundle-sheath of other C$_4$ plants may be as high as 1000 $\mu$M (four times ambient). However, modeling has shown that bundle-sheath O$_2$ concentrations can be several-fold greater than ambient O$_2$ concentration without large effects on CO$_2$ assimilation (von Caemmerer and Furbank, 1999). Two features will tend to mitigate O$_2$ evolution in the bundle-sheath. First, reduction of glycerate-3-P in the mesophyll will result in a decrease in the requirement for reductant, and hence photosynthetic O$_2$ evolution, in the bundle-sheath. Second, the photosynthetic carbon fluxes through the mitochondria in NAD-malic enzyme and PCK-types are 10- to 20-fold higher than rates of respiration in other tissues. Such high rates of respiration may lower the O$_2$ concentration in the bundle-sheath.

### IV. Intracellular Metabolite Transport in C$_4$ Plants

Leaf organelles in C$_4$ plants share the translocators of organelles in leaves of C$_3$ plants (Chapter 6, Flügge), but also contain translocators with unique, or considerably altered, kinetic properties. The chloroplasts of C$_4$ plants also possess a peripheral reticulum, a membrane system of anastomosing tubules contiguous with the inner membrane of the chloroplast envelope. This is generally more highly developed in the mesophyll chloroplasts, particularly in C$_4$ dicots, although it is also prominent in the bundle-sheath chloroplasts of the C$_4$ Cyperaceae (Dengler and Nelson, 1999). It function remains unexplored, although it may serve to increase the area of the chloroplast envelope so as to facilitate transport (Laetsch 1974; Hatch and Osmond 1976).

#### A. Mesophyll Chloroplasts

During photosynthesis in C$_4$ plants, the mesophyll chloroplasts import glycerate-3-P, deriving from the bundle-sheath, and export triose-P, and the bundle sheath chloroplasts export glycerate-3-P to the mesophyll and import triose-P that has been reduced by the mesophyll chloroplasts. This is quite different from chloroplasts of a C$_3$ plant, which export triose-P in exchange for Pi (Chapter 6, Flügge). In addition, the mesophyll chloroplasts also catalyze the export of PEP, formed in the chloroplast by the action of pyruvate P$_i$ dikinase, in exchange for P$_i$ to sustain PEP carboxylase in the cytosol (Fig. 2). Exchange of PEP, P$_i$, glycerate-3-P and triose-P occurs on a common Pi translocator in the chloroplast envelope of C$_4$ plants. The C$_3$ mesophyll Pi translocator is very similar to the C$_3$-type phosphate translocator, with between 83 and 94% identity in amino acid residues. Minor changes in amino acid sequence have occurred to extend the substrate specificity of the C$_3$ phosphate translocator to recognize PEP in C$_4$ plants (Fischer et al., 1994). Ohnishi et al., (1989) studied the Pi translocator in mesophyll chloroplasts of Panicum miliaceum (NAD-ME type). The affinity of the translocator for glycerate-3-P and DHAP was lower.
than for Pi, in contrast to C₃ chloroplasts, in which it is generally higher by a factor of two- to three-fold. Light stimulated the uptake of Pi in mesophyll chloroplasts, an effect not observed in bundle-sheath chloroplasts (and not previously reported for chloroplasts from plants). Light also increased the for glycerate-3-P and DHAP in the mesophyll chloroplasts, further decreasing the affinity of the translocator for these compounds. The increased values for these metabolites in the light would permit the exchange of PEP and Pi in the presence of the very high concentrations of glycerate-3-P and DHAP which are attained in the mesophyll cytosol and chloroplasts (Tables 1 and 2).

Chloroplasts transport pyruvate on a specific carrier in both C₃ and C₄ plants, but the translocator is much more active and is light-dependent in mesophyll chloroplasts of C₄ plants (Flügge et al., 1985). Pyruvate transport appears to be driven by an H⁺ gradient in NADP-malic enzyme grasses of the Arundineleae and Andropogoneae and a gradient in other monocots and all dicots (Ohnishi and Kanai, 1990; Ohnishi et al., 1990; Aoki et al., 1992, 1994; Heldt and Flügge, 1992; Murata et al., 1992). It would appear that the H⁺ taken up by H⁺/pyruvate cotransport is released in vivo together with PEP (which is generated as PEP³⁻, but transported via the Pi translocator as PEP³⁻)(Aoki and Kanai, 1995; Fig. 2). In the Na⁺-type, pyruvate transport may occur by Na⁺-dependent acceleration of pyruvate-H⁺ cotransport (Aoki and Kanai, 1997). Interestingly, all C₄ plants require Na⁺ as an essential micronutrient (Brownell and Crossland 1972). The Na⁺-dependence of the pyruvate transporter in some C₄ species may help explain this requirement in some species, although some other process in the conversion of pyruvate to PEP must also show Na⁺-dependence in NADP-ME species (Brownell et al., 1991).

The dicarboxylates (oxaloacetate, malate, 2-oxoglutarate, glutamate and aspartate) are transported across the chloroplast envelope in a carrier-mediated mode. These compounds undergo counter-exchange on the dicarboxylate translocator. The Kᵢ for uptake of a particular dicarboxylic acid is similar to the Kᵢ for the inhibition of uptake by other dicarboxylates. In maize, for example, the Kᵢ (0.3 mM) for oxaloacetate inhibition of malate transport is comparable to the Kᵢ (0.5 mM) for malate uptake (Day and Hatch, 1981). In NADP-ME species, and to some extent in PCK-type species, oxaloacetate generated by PEP carboxylase must be taken up into the chloroplasts for reduction to malate. Oxaloacetate uptake would clearly not occur when oxaloacetate concentrations are several orders of magnitude less than malate concentrations, as occurs in NADP-ME plants such as maize, in which OAA concentrations are probably less than 50 μM. There is, therefore, a quite separate and very active oxaloacetate carrier in maize mesophyll chloroplasts (Kᵢ 45 μM) which is little affected by malate (Kᵢ 7.5 mM)(Hatch et al., 1984).

B. Bundle Sheath Organelles

Although transport processes across the envelope of C₄ mesophyll chloroplasts are now adequately characterized, relatively little is known of transport into the bundle sheath chloroplasts, largely because of the difficulty of isolating these intact and in appreciable quantities from any C₄ plants. Bundle sheath chloroplasts have a phosphate translocator like that of C₃ and C₄ mesophyll chloroplasts (Chapter 6, Flügge). However, bundle-sheath chloroplasts are unusual because they must export glycerate-3-P and import triose-P at high rates. In C₃ plants, glycerate-3-P is not exported by chloroplasts to any great extent because it is transported by the translocator as the glycerate-3-P²⁻ ion, whereas glycerate-3-P³⁻ is the form that predominates at the pH which occurs in the illuminated stroma (Heldt and Flügge, 1992). It is possible that the stromal pH is lower in bundle sheath chloroplasts or that glycerate-3-P within the chloroplast reaches such high internal concentrations that transport of glycerate-3-P²⁻ from the chloroplast becomes inevitable. Ohnishi et al., (1989) studied
the Pi translocator in bundle-sheath chloroplasts of Panicum miliaceum (NAD-ME type). The chloroplast Pi translocator from the bundle-sheath had a surprisingly high affinity for PEP. Bundle-sheath chloroplasts had a higher $K_m$ (Pi) (0.33 mM) compared with the mesophyll chloroplasts (0.14 mM), but the $K_i$ (glycerate-3-P) was little different from that of the mesophyll chloroplasts.

Little is known about transport of organic and amino acids into bundle sheath chloroplasts. The bundle sheath chloroplasts of Panicum miliaceum (NAD-ME type) show a slow carrier-mediated uptake of pyruvate which has very similar characteristics to the carrier in wheat and pea chloroplasts and which is not light-stimulated, in contrast to mesophyll chloroplasts, but this could have a function unconnected to photosynthesis. In maize, malate decarboxylation by bundle-sheath chloroplasts is appreciably enhanced by aspartate and glutamate. As aspartate and glutamate are not required by NADP-ME, it has been suggested that a carrier specific for malate uptake is present which depends upon the presence of aspartate for maximum activity or that, if malate uptake is facilitated by an exchange carrier similar to that in C$_4$ plants or C$_4$ mesophyll chloroplasts, rapid aspartate or glutamate uptake via another carrier might allow malate to exchange with a concomitant efflux of aspartate or glutamate. In either case, the existence of specialized dicarboxylate carriers in maize bundle-sheath chloroplasts is implicated (Boag and Jenkins, 1985, 1986). Pyruvate has also been shown to have a stimulatory effect on malate transport into bundle-sheath chloroplasts of maize (Kanai and Edwards, 1999). Bundle-sheath chloroplasts also have a glycolate transporter (Ohnishi and Kanai, 1988), although the glycerate formed in the photorespiratory pathway is subsequently metabolized in the mesophyll.

An even greater area of uncertainty is metabolite transport into the mitochondria of bundle-sheath cells. In NAD-ME-type species, decarboxylation of C$_4$ acids by both isolated bundle-sheath cells and by mitochondria requires aspartate and 2-oxoglutarate. These act as a source of oxaloacetate, which is then endogenously reduced to malate, reductant being regenerated by the oxidation of malate by NAD-malic enzyme (Chapter 18, Furbank et al.). However, external malate and phosphate are also required (Furbank et al., 1990b). It has been proposed that the requirement for malate results from a counter-exchange of external malate for internal Pi (Day and Wiskich, 1984), followed by exchange of external 2-oxoglutarate and internal malate. Pi would then be taken up again by a Pi/OH antiporter or Pi/H$^+$ symporter (Furbank et al., 1990b; Douce et al., 1997; Fig. 3). This link between Pi and C$_4$ acid decarboxylation could provide feedback from sucrose synthesis (which releases Pi) to CO$_2$ fixation in the bundle-sheath. Similarly, sensitivity to malate could link CO$_2$ fixation in the mesophyll to rates of decarboxylation in the bundle-sheath, in a manner analogous to the regulation of malate decarboxylation by aspartate in NADP-ME plants (see above). In PCK-type species, in which malate is decarboxylated in the mitochondria and pyruvate and ATP exported to the cytosol, it is presumed that a similar dependence of malate transport on Pi obtains because malate decarboxylation is highly dependent on Pi, in addition to the requirement to generate ATP for the reaction catalysed by PCK (Carnal et al., 1993). Mitochondria are also likely to be involved in aspartate metabolism in NADP-ME type plants, as aspartate aminotransferase in the bundle-sheath of maize is mainly located in the mitochondria (Chapman et al., 1981; Wingler et al., 1999).

A mitochondrial 2-oxoglutarate/malate translocator has been cloned from leaves of Panicum miliaceum. It is highly homologous with the bovine 2-oxoglutarate/malate translocator and the recombinant protein transports malate, citrate and 2-oxoglutarate.
tarate when incorporated into liposomes (Taniguchi and Sugiyama, 1996).

V. Concluding Remarks

High rates of both intra- and inter-cellular transport of metabolites are crucial to the operation of CO₂ assimilation by C₄ plants. Both the chloroplasts and mitochondria are involved in intercellular metabolite transport during photosynthesis in most C₄ plants. Most of these transporters remain to be characterized at the molecular level and their interactions understood. This is particularly important in view of the many attempts to genetically modify photosynthesis to introduce C₃ characteristics into C₄ plants. It is also evident that C₄ photosynthesis involves intercellular transport via plasmodesmata, which involves considerable structural modification of the mesophyll/bundle-sheath interface. There is still much to be understood about the factors which affect plasmodesmatal development and permeability (Chapter 11, Schober et al.). In addition, there are other anatomical differences between C₃ plants, for example, the centripetal versus centrifugal location of chloroplasts within the bundle-sheath and the presence of a double bundle-sheath in NAD-ME and PCK types (Dengler and Nelson, 1999) whose consequences for intra- and inter-cellular transport are likely to be profound but which have not been evaluated.

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Summary

Development of a fully functional C_4 leaf requires modification of the C_3 ground state at several hierarchical levels, including vein spacing in whole leaves, cell division and cell enlargement patterns within the photosynthetic tissue precursors, ultrastructural changes in bundle sheath (BS) and mesophyll (M) cells, and regulation of gene expression through differential transcription, translation, and protein turnover. Both spatial and temporal patterns of BS and M cell differentiation indicate that cell position is the primary determinate of photosynthetic cell fate and that cell-to-cell communication is essential for interpretation of position. The nature of signaling pathways in C_4 development is unknown, but recent demonstrations of macromolecular trafficking through plasmodesmata and of the role of receptor-like kinases in tissue pattern formation provide clues to the mechanisms co-opted by C_4 plants. The regulation of photosynthetic gene expression occurs at two
levels (which may coincide temporally) in C₄ plants: gene expression occurs first in an organ- and cell-specific way only in those cells where chloroplasts develop and replicate to high numbers; the positional signals involved are likely common to both C₁ and C₄ plants. Second, gene expression patterns that are restricted to either BS or M cells occur; these patterns are limited to C₄ plants, but may use some of the same positional signals to achieve differentiation of BS and M cells. Experimental approaches that are providing insights into the developmental regulation of these genes include reporter gene fusions to identify cis-acting sequences and screens to identify mutations that disrupt C₄ developmental patterns. The evidence to date indicates that each C₄ gene uses its own regulatory mechanism to achieve cell specificity; even if all cell-specific genes respond to the same signal, these signals appear to activate different mechanisms which in turn activate or repress the expression of C₄ genes.

I. Introduction

C₄ photosynthesis requires developmental regulation at several levels of leaf organization. In all plants, regardless of photosynthetic pathway, the internal architecture of the leaf is determined in a series of hierarchical processes. When leaves are formed by the shoot apical meristem, the surface layer, precursor to dermal tissue system, is distinct from internal layers, based on differential planes of cell division (Esau, 1965). Delimitation of the vascular tissue system from other internal tissues occurs during very early stages of leaf expansion. Formation of individual tissue types within each of the three tissue systems (dermal, ground, and vascular) involves distinctive planes of cell division and enlargement to give rise to each tissue in its appropriate position. Cell differentiation requires differential enlargement and the expression of cell-specific biochemical and structural characteristics, including cell wall and organelle components. Although these processes occur at different levels of organization (leaf, tissue system, tissue, cell, organelle), they are coordinated during development so that a characteristic temporal sequence is followed and elements are formed in characteristic spatial patterns. Formation of a fully functional C₄ leaf requires modification of the C₁ ground state developmental programs that determine all of these processes, specifically: 1) overall tissue pattern within the leaf, 2) cell pattern within tissues, and 3) biochemical compartmentation and structural cell differentiation (Fig. 1). Although far more is known about cell specific enzyme expression on a molecular and genetic level than any other aspect of C₄ photosynthesis, this chapter reviews alterations to all three levels of leaf organization that are required for development of the C₄ syndrome, discusses the interactions between these levels, and identifies experimental approaches that will advance our understanding of the developmental basis of C₄ photosynthesis.

A. Relationship between C₄ Biochemistry and Structure

In C₄ plants, all chloroplast-containing cells, including leaf M and BS cells, are equivalent in terms of photosynthetic pathway, although BS cells typically have fewer and smaller chloroplasts (Fig. 1). In both cell types atmospheric CO₂ is assimilated by carboxylase activity of the chloroplast enzyme RuBPCase and reduced by the activities of other Calvin cycle enzymes, using ATP and NADPH produced by the light reactions to form three carbon triose phosphate molecules and, ultimately, sucrose and starch. As a consequence of the oxygenase activity of RuBPCase, C₃ photosynthetic tissues also experience the energetically wasteful process of photorespiration. C₄ plants effectively suppress photorespiration by a spatial separation of the C₃ Calvin cycle activity in BS cells and of the C₄ cycle in M cells. The C₄ cycle acts as a biochemical CO₂ pump, concentrating CO₂ ten-fold over atmospheric concentrations and thus greatly reducing the oxygenase activity of RuBPCase (Edwards and Walker, 1983; Hatch, 1987; Furbank and Taylor, 1995). CO₂ is initially fixed within M cells by
mentation of initial CO$_2$ fixation steps in M cells and the subsequent carbon reduction steps in BS cells, the two photosynthetic cell types of C$_4$ plants differ strikingly in enzyme complement. Since all of these enzymes are either components of the Calvin cycle or participate in other nonphotosynthetic functions within the plant, C$_4$ photosynthesis depends on regulatory mechanisms that produce these cell-specific expression patterns (Moore, 1982; Hermans and Westhoff, 1990; Ku et al., 1996). In addition, C$_4$ forms of these enzymes may differ from their C$_3$ counterparts by having much higher activities and levels of expression, different kinetic properties, and different patterns of light-regulated expression and intracellular localization (Bauwe and Chollet, 1986; Harpster and Taylor, 1986; Hatch, 1987; Hatch and Burnell, 1990; Schäßfner and Sheen, 1992; Kubicki et al., 1994).

C$_4$ photosynthetic biochemistry is accompanied by a distinctive pattern of leaf anatomy historically referred to as ‘Kranz anatomy’ (Fig. 1; Brown, 1975; Hatch, 1987). Despite the great diversity in basic leaf architecture in the plant families in which C$_4$ photosynthesis occurs, certain anatomical features are invariably associated with the pathway and are regarded as being essential for its operation. These are: 1) structural specialization of the two types of photosynthetic cells, 2) spatial configuration of these tissues that maximizes contact between M and BS cells, and 3) features that limit the rate of leakage from the bundle sheath cells (Hattersley and Browning, 1981; Hattersley, 1984; Hattersley and Watson, 1992). Although unusual anatomical types occur (Dengler and Nelson, 1998), typical C$_4$ BS cells are large in comparison to their counterparts, have more numerous chloroplasts, and an asymmetric arrangement of cytoplasmic components. M cells are similar in size to those of C$_3$ species and, like them, have peripherally arranged chloroplasts (Fig. 1C). In C$_4$ plants, BS and M cells are intimately associated; M cells are usually elongate in a radial direction, an arrangement that permits each cell to be in contact with a BS cell (Fig. 1B). BS cells are typically associated with leaf veins and thus veins are closer together in C$_4$ species (Fig. 1A). Numerous plasmodesmata extend across the shared BS/M cell wall, providing an extensive pathway for the diffusion of metabolites (Fig. 1C). The cell wall at the BS/M cell interface is often highly modified: a suberin lamella, thought to be impermeable to CO$_2$, is deposited in the BS portion of the wall of many C$_4$
species. The volume of intercellular airspace is lower in \( C_4 \) species than in related \( C_3 \) species, and both the shape of BS cells and the arrangement of M cells reduce exposure of the BS cell surface to intercellular airspace, thus reducing the pathway for \( \text{CO}_2 \) leakage (Dengler et al., 1994).

The \( C_4 \) pathway is an adjunct to the universal \( C_3 \) pathway and depends not only on novel patterns of photosynthetic enzyme expression, but also on cell-specific changes in patterns of cell enlargement, cell wall modifications, chloroplast division and growth, and placement of organelles within the cell. The genetic programs that guide these developmental processes are all present in some form in \( C_1 \) plants, so that the primary requirement for the development of Kranz anatomy is their position-specific regulation. In addition, the planes of cell division and of directional cell enlargement are modified during formation of \( C_4 \) mesophyll tissue, and formation of leaf vascular pattern is altered to result in the close vein spacing of \( C_4 \) plants. These diverse developmental events must be regulated in a coordinated way as each leaf expands and matures. Thus the development of \( C_4 \) photosynthesis offers an opportunity to address questions not only of the mechanisms of cellular differentiation, but also how developmental pathways and regulatory mechanisms are coupled across organizational levels.

**B. Multiple Evolutionary Origins**

The evolutionary pattern of \( C_4 \) photosynthesis makes questions about its developmental regulation particularly interesting and challenging. The taxonomic distribution of \( C_4 \) plants indicates that this photosynthetic pathway evolved numerous times during the diversification of flowering plants, \( C_4 \) photosynthesis occurs in genera of at least 16 dicotyledon families, including the Amaranthaceae, Asteraceae, Chenopodiaceae, Euphorbiaceae, and Portulacaceae (Downton, 1975; Ragavendra and Das, 1978). Although \( C_4 \) species are relatively numerous in the Chenopodiaceae and Euphorbiaceae, each of the 16 dicot families contains only a few genera (in some cases only a single genus) with \( C_4 \) species, while the remaining genera of the family are wholly \( C_3 \). The predominance of \( C_3 \) photosynthesis in each family and the lack of a common ancestor having the \( C_4 \) photosynthetic pathway is indicative of a series of independent evolutionary origins for \( C_4 \) photosynthesis. Only two families of monocotyledons, the grasses (Poaceae) and sedges (Cyperaceae) have \( C_4 \) species, but these are more numerous. It is estimated that one-half (5,000 species) of all grass species are \( C_4 \), and phylogenetic analyses of the family indicate at least four different origins for subgroups with \( C_4 \) photosynthesis (Hattersley and Watson, 1992; Kellogg, 1996; Sinha and Kellogg, 1996). In addition, the complex pattern of photosynthetic pathway variation within the grasses also indicates that reversals from \( C_4 \) to \( C_3 \) photosynthesis have occurred more than once (Hattersley, 1987). The phylogeny of the sedge family is less well understood, but it is estimated that about 40% of a total 4,000 species are \( C_4 \) and that the pathway has arisen three to four times (Bruhl, 1995).

These evolutionary patterns raise important questions for understanding some aspects of the developmental regulation of the \( C_4 \) syndrome: if there have been 20 or more separate evolutionary origins of \( C_4 \) photosynthesis, are there as many different molecular mechanisms that regulate each aspect of the \( C_4 \) syndrome or is the spectrum of possibilities limited? Do genes co-opted for \( C_4 \) photosynthesis share common regulatory mechanisms so that a suite of genes is expressed under the control of a ‘master switch?’ Are the biochemical variants of \( C_4 \) photosynthesis always equivalent; for example, is the same mechanism used for the regulation of BS-specific NAD-ME expression in a grass and a dicotyledon? The answers to these questions require the methodological approaches of comparative biology where developmental regulation of biochemical and anatomical traits is compared between closely related groups of \( C_3 \) and \( C_4 \) species (Hattersley and Watson, 1992; Kellogg, 1996; Monson, 1996). While the primary goal of comparative studies is to understand evolutionary diversification, their findings may also be useful for understanding some aspects of developmental regulation. Our goals in this review are to identify both the structural and biochemical features that are essential for the operation of the \( C_4 \) pathway, to review our current understanding of their developmental regulation, and to discuss future prospects for resolving outstanding questions. Although we focus on certain model organisms, we also speculate on whether different evolutionary events arrived at common developmental mechanisms or whether substantial diversity exists in the developmental regulation of \( C_4 \) photosynthesis.
II. Formation of Tissue Pattern

A. Vein Spacing

Compartmentation of the primary carbon assimilation and photosynthetic carbon reduction steps of the $C_4$ pathway imposes a physiological requirement for the rapid flux of metabolites between BS and M cells. In $C_4$ grass species, ten or more M cells may intervene between the BS cells of adjacent veins, while in $C_3$ grasses no M cell is more than one cell removed from the bundle sheath and, in fact, most M cells are in direct contact with a BS cell (Fig. 1A,B; Hattersley and Watson, 1975). This requirement results in the reduction of total mesophyll tissue volume in the leaves of $C_4$ species in comparison to their $C_3$ relatives (Hattersley, 1984; Dengler et al., 1994) and, because BS tissue is typically (but not universally) associated with leaf veins, in the close spacing of adjacent leaf veins (Crookston and Moss, 1974; Kawamitsu et al., 1985; Dengler et al., 1994). Sinha and Kellogg (1996) concluded that, given the diversity in positional relationships between BS and M cells and in accumulation patterns of photosynthetic enzymes other than RuBPCase and PEPCase, interveinal distance was one of the few consistent characteristics that consistently distinguishes $C_4$ from $C_3$ species. Thus, evolution of $C_4$ photosynthesis has universally required modification of the regulatory pathways that determine vein pattern early in leaf development.

B. Vein Pattern Ontogeny

Precursors of dermal and ground tissues are present from leaf inception, but precursors of vascular tissues become established during early stages of leaf development. It is only after the establishment of vascular pattern that BS and M cells become delimited in relation to the veins (Langdale and Nelson, 1991; Nelson and Dengler, 1992). In dicotyledons, the most common leaf vascular pattern becomes established in three discrete phases: 1) the midvein provascular strand develops in continuity with the stem vasculature, extending from the stem into a new leaf primordium, 2) secondary vein provascular strands grow progressively from the midvein toward the margin concurrent with the formation of the leaf lamina, and 3) minor vein provascular strands form a network of small veins between the secondary veins, usually in a basipetal direction (Fig. 2A-C). Minor vein formation coincides with the early stages of leaf expansion and is coordinate with it so that as new veins are intercalated between older ones, the mean distance between adjacent veins tends to remain constant. These last stages of vascular pattern formation are those that must be modified during leaf development in a $C_4$ dicotyledonous species: the developmental programs that determine vein spacing patterns must be altered to result in the short interveinal distance in the leaves of $C_4$ species (Fig. 2D). Development of the striate venation pattern of grass leaves such as maize differs in several respects from dicotyledons (Evert et al., 1996; Dengler et al., 1997). The largest longitudinal veins initially appear in isolation from stem vasculature and connections between these veins and the stem vascular bundles occur only secondarily. As the grass leaf grows in width, new longitudinal veins are intercalated between previously formed veins, maintaining a constant mean distance between adjacent veins (Fig. 2E-G). This
process is modified in C₄ species so that formation of small longitudinal veins is prolonged or accelerated, resulting in a greater number of more closely spaced veins (Fig. 2H).

C. Regulation of Vein Pattern Formation

Since C₄ species are distinguished from C₃ species by closer vein spacing, a key step in the evolution of a C₄ species is an alteration in the regulatory pathways that determine vascular pattern. At present, very little is known about the genetic and molecular control of C₄ vein spacing. A screen of the M2 generation from EMS-mutagenized seed of the C₄ grass Panicum maximum produced several putative mutants with an increase in both interveinal distance and number of M cells intervening between adjacent veins (Fladung, 1994). One of these, the lisl mutation, had lower photosynthetic rates and more C₃-like CO₂ compensation points, indicating that vein spacing is critical for the efficient operation of the C₄ pathway. Unfortunately, it was not possible to carry these putative mutants to advanced generations, so the molecular and physiological roles of lisl in determining C₄ vein spacing is unknown.

Other experimental and genetic evidence points to the importance of the polar transport of auxin in the formation of vascular strands. Based on careful observations of regeneration of vascular tissue around mechanically induced wounds in stems and leaves in response to auxin application, Sachs (1981, 1989, 1991) proposed that canalization of auxin flow occurred through the following steps: 1) initially all cells in a developmental field are equivalent transporters of auxin, 2) gradually certain cells become better auxin transporters and, through positive feedback, their capacity to transport auxin increases with auxin flux, and 3) the cells transporting auxin are induced to differentiate as M cells, as well as a possible change in the pattern of enlargement of ground meristem cells as they differentiate as M cells, as well as a possible change in cell division pattern (Fig. 3B). Such alterations in plant cell division and cell expansion are generally anticipated by changes in cytoskeleton components: cell division plane reflects the orientation of the preprophase band of microtubules and microfilaments, and the direction of cell elongation is perpendicular to the predominant orientation of cytoskeleton elements (Lloyd et al., 1992; Cyr, 1994). The fass and ton mutations of Arabidopsis affect both aspects of the cytoskeleton: cortical microtubules are disorganized and preprophase bands are not formed (Torres-Ruiz and Jurgens, 1994; Traas et al., 1995). Although tissues of mutant plants occur in the correct positions, they show defective planes of cell division and cell enlargement, indicating that these genes are necessary for normal formation of cell patterns within tissues. These mutant phenotypes are expressed throughout the plant, indicating that they are not regulated in an organ- or vascular pattern formation is dependent on the polar flow of auxin. Similarly, mutations in the LOPPED (LOP) gene result in a greatly reduced leaf lamina that lacks minor venation, but has an oversized or twinned midvein (Carland and McHale, 1996). Mutant plants have normal levels of free auxin, but reduced capacity for auxin transport, also pointing to a role for the polar transport of auxin in normal vascular pattern formation. If auxin transport characteristics are indeed crucial for vascular pattern formation, it is likely that mechanisms associated with the positive feedback loop postulated by Sachs became modified during the evolution of C₄ from C₃ species. If perception of auxin level or response to a specific auxin signal is altered, then canalized pathways for auxin transport might arise at closer spacing, resulting in characteristic C₄ vein densities in mature leaves.

III. Cell Pattern within Tissues

The physiological requirement for contact between M and BS cells has resulted in an alteration of the layered arrangement of C₃ mesophyll tissue to the typical C₄ arrangement in which M cells are arrayed in a radial pattern in relation to the leaf veins (Fig. 1B). The radial arrangement of C₃ mesophyll is acquired relatively late during leaf development (Dengler et al., 1995) and requires a change in the pattern of enlargement of ground meristem cells as they differentiate as M cells, as well as a possible change in cell division pattern (Fig. 3B). Such alterations in plant cell division and cell expansion are generally anticipated by changes in cytoskeleton components: cell division plane reflects the orientation of the preprophase band of microtubules and microfilaments, and the direction of cell elongation is perpendicular to the predominant orientation of cytoskeleton elements (Lloyd et al., 1992; Cyr, 1994). The fass and ton mutations of Arabidopsis affect both aspects of the cytoskeleton: cortical microtubules are disorganized and preprophase bands are not formed (Torres-Ruiz and Jurgens, 1994; Traas et al., 1995). Although tissues of mutant plants occur in the correct positions, they show defective planes of cell division and cell enlargement, indicating that these genes are necessary for normal formation of cell patterns within tissues. These mutant phenotypes are expressed throughout the plant, indicating that they are not regulated in an organ- or
Chapter 20  Development of C₄ Photosynthesis

A. Vein spacing

B. Tissue pattern

C. Cell differentiation - structure

D. Cell differentiation - biochemistry

E. Cell differentiation - gene regulation

Fig. 3. Temporal sequence (and presumed hierarchy of genetic control) of C₄ development. A. Vein spacing. B. Cell pattern within tissue, involving patterns of cell division and enlargement. C. Structural aspects of cell differentiation. D. Biochemical aspects of cell differentiation. E. Differential gene expression. Cell-cell signaling pathways through plasmodesmata and/or putative membrane-bound receptor-like kinases may be involved at each level. Note differing patterns between M cells adjacent to BS (M1) and more distant M (M2) cells.

cell-specific manner.

Several genes that have a role in chloroplast development also affect cell elongation in a tissue-specific manner. For instance, Arabidopsis plants with a mutation in the PALE CRESS (PAC) gene have defective chloroplasts, and normal elongation of palisade mesophyll during leaf development is disrupted (Reiter et al., 1994). In mutant plants mesophyll layers form normally, but the distinctive elongate palisade cell shape is never fully developed, indicating genetic regulation over initial cell elongation and maintenance of elongate shape during development. Similarly, the differentiation and greening (dag) mutation of Antirrhinum and defective chloroplasts and leaves-mutable (dcl-m) mutation of tomato block elaboration of internal chloroplast membranes and also repress normal elongation of palisade mesophyll (Chatterjee et al., 1996; Keddie et al., 1996). These mutant phenotypes indicate that normal palisade cell elongation requires a plastid-derived signal and, while there is no direct evidence, such a signaling pathway presumably would interact with the cytoskeleton to produce differential cell elongation. Although these genes have been identified in C₄ species, these studies may have identified signaling pathways that could be involved in C₄ mesophyll-specific elongation patterns. In C₄ plants any regulatory mechanism that links chloroplast development, cytoskeletal organization, and cell enlargement must act downstream of the positional signals that identify M cells. Such genes must be among the first to be specifically activated in the development of a C₄ leaf.

IV. Bundle Sheath and Mesophyll Cell Structural Differentiation

A. Chloroplast Dimorphism

The chloroplast complements of mature BS and M cells differ strikingly: BS cells typically have greater numbers and larger chloroplasts, leading to a higher proportion of cell volume occupied by the plastome (Laetsch, 1974; Kirchanski, 1975; Dengler et al., 1986, 1996; Liu and Dengler, 1994; Ueno, 1996). Individual BS and M cell chloroplasts differ in shape as well as size: M cell chloroplasts tend to be elliptical in section, while BS chloroplasts are more likely to have a flattened profile. Also, starch grains are usually present in mature BS chloroplasts, but reduced or absent in M chloroplasts. The most dramatic differences between BS and M cell chloroplasts lie in the development of internal chloroplast membranes. In NADP-ME species, BS cell chloroplast thylakoids form only a small number of rudimentary grana,
while M cell chloroplasts have numerous large grana (Brangeon, 1973; Laetsch, 1974; Kirchanski, 1975; Ueno et al., 1988; Dengler et al., 1996). Reduced granal stacking in BS chloroplasts is correlated with reduced abundance of PS II polypeptides in NADP-ME type grasses (Schuster et al., 1985; Meierhoff and Westhoff, 1993; Kubicki et al., 1994). In NAD-ME type species, grana development is similar in BS and M cell chloroplasts, but chloroplasts are still dimorphic, large in BS and small in M cells (Laetsch 1974; Dengler et al., 1986; Liu and Dengler, 1994).

Another conspicuous difference between BS and M cell chloroplasts is the degree of development of a peripheral reticulum from the inner chloroplast envelope, although the pattern of variation is not consistent among C₄ types. In a number of C₄ dicotyledons, peripheral reticulum is better developed in M than BS cells (Laetsch, 1974; Chapman et al., 1975; Sprey and Laetsch, 1978; Liu and Dengler, 1994), but in C₄ species of the Cyperaceae, peripheral reticulum is more prevalent in BS cells, forming extensive arrays of vesicles or anastomosing tubules at the periphery of the chloroplasts (Ueno et al., 1988). Thus, C₄ developmental programs must involve regulation of chloroplast number per cell, chloroplast enlargement, elaboration of internal membranes to form grana and peripheral reticulum, and expression and activity of starch synthetic pathways (Fig. 3C).

Chloroplast number per cell typically increases about ten-fold as cells enlarge during leaf development (Ellis and Leech, 1985; Boffey, 1992). Proplastids divide as cells divide, but with the cessation of mitosis, enlarging plastids may continue to divide until the mature number of chloroplasts per cell is reached. Regulation of plastid replication and subsequent enlargement is cell-specific, since mesophyll, parenchymatous bundle sheath, vascular parenchyma, and epidermal guard cells all differ in chloroplast number and size in mature leaves (Pyke and Leech, 1994). Several nuclear genes that affect this process have been identified in Arabidopsis (Pyke and Leech, 1992, 1994; Pyke et al., 1994). Mutations in ACCUMULATION AND REPLICATION OF CHLOROPLASTS (ARC) genes result in a reduction in number of chloroplasts, but there is a compensating increase in chloroplast size so that total complement per cell is similar in mutants and wildtype. Chloroplast ultrastructure is affected only slightly, indicating that arc mutations are primarily defects in the plastid division process (Pyke et al., 1994). ARC genes also appear to have cell-specific effects: in wildtype leaves, chloroplasts of the parenchymatous bundle sheath (equivalent of C₃ BS cells) are smaller than chloroplasts of M cells, but in arc1 mutants they are larger (Pyke and Leech, 1994). ARC genes may be among those which must be regulated differently in C₄ compared with C₃ plants. In C₄ species, chloroplast division must be upregulated during development of C₄ BS cells, but, since BS cells typically have both more and larger chloroplasts, chloroplast enlargement must also be regulated, resulting in a total chloroplast complement that is dramatically larger than that of C₃ BS and M (or C₄ M) cells.

Development of the dimorphic chloroplast ultrastructure of BS and M cells is best known for maize and other C₄ grass species where there is a longitudinal gradient of cell maturation stages in cell files produced by the basal elongation zone (Laetsch and Price, 1969; Brangeon, 1973; Kirchanski, 1975; Dengler et al., 1996). At the youngest stages, BS and M cell proplastids are morphologically identical, with numerous starch grains and rudimentary grana. As plastids in both cell types increase in size, stroma thylakoids become more extensive and appressed regions become more frequent; starch grains disappear during this growth phase. When BS chloroplasts reach about 50% of their mature size, appressed thylakoids become disassociated, reducing the number and extent of grana, despite the continued growth of stroma thylakoids. Loss of grana is correlated with reduced activity of PS II as indicated by photoreduction of tetrazolium blue (Wrischer, 1989) and by chlorophyll a fluorescence measurements (J. A. Langdale, personal communication). At late developmental stages, conspicuous starch grains appear in BS cell chloroplasts, but are less prevalent in M cell chloroplasts. Mutations in Bundle sheath defective-mutable genes (Bsd1-m1 and Bsd2-m1) affect chloroplast ultrastructure in BS and M cells differentially, essentially arresting BS chloroplast development at an early stage and/or retarding growth patterns (Langdale and Kidner, 1994; Roth et al., 1996). Although Bsd1-m1 and Bsd2-m1 appear to play a primary role in rbcL transcript accumulation, these genes also appear to have at least an indirect role in regulating the divergent developmental pathways of BS and M cell chloroplasts.

B. Asymmetric Placement of Organelles

BS cell organelles typically have an asymmetric...
placement within the cell, while M cell organelles are symmetrically distributed, as is characteristic for other vacuolate parenchyma cells. In NADP-ME type species, BS cell chloroplasts, mitochondria, microbodies and nuclei are located in a centrifugal position, toward the mesophyll side of the cell, while in NAD-ME type species, organelles are located in a centripetal position, adjacent to the vascular tissue (Gutierrez et al., 1974; Hatch et al., 1975). The organelles of immature BS cells in the basal extension zone of grass leaves have a symmetrical distribution around a central vacuole; as cells cease elongation at the distal end of the extension zone, organelles migrate toward their final position (Miyake and Yamamoto, 1987; Dengler et al., 1996).

Both microtubules and microfilaments are known to be involved in some types of chloroplast movements (Williamson, 1986); however, chloroplast-associated microtubules were not observed in leaf blades of the NAD-ME grass Eleusine corocana although they were present in the cortical cytoplasm of the same cells (Miyake and Yamamoto, 1987). Evidence for the involvement of actin microfilaments in chloroplast migration in developing Eleusine leaf blades was obtained from a series of experimental treatments of isolated elongating leaf blades: cytochalasin B, an inhibitor of microfilaments, almost completely blocked chloroplast migration, while colchicine, an inhibitor of microtubule assembly, had no effect (Miyake and Nakamura, 1993). Chloroplast movement was also inhibited by cycloheximide, an inhibitor of cyt protein synthesis, indicating that production of actin and possibly force-generating ATPases may be required for migration. Most growth hormone treatments had no effect on this process, but chloroplast movement was inhibited by auxin treatment, indicating that auxin itself or the physiological status of elongating tissues might prevent migration. These processes do not appear to be specific for chloroplasts themselves because nuclei, mitochondria and microbodies always moved with the chloroplasts. Light is likely an important part of the signaling pathway for the asymmetric placement of organelles, since dark treatment prevented chloroplast migration in Eleusine and is required for organellar movement in other systems (Haupt, 1982). Organelle position appears to be fixed once BS cells have differentiated, although the striking centrifugal position of BS cell chloroplasts was lost in response to severe water stress in maize (Lal and Edwards, 1996).

C. Suberin Lamella

The vascular bundles of all grasses, both C₃ and C₄, are surrounded by an inner sheath layer, the mestome sheath (Esau, 1965). Mestome sheath cells are non-chlorenchymatous and have thickened cell walls that are modified by deposition of a layer of suberin (O’Brien and Carr, 1970). In NADP-ME type grasses such as maize, the outer chlorenchymatous bundle sheath layer is absent and mestome sheath cells are modified as BS cells (Dengler et al., 1985). In these species, the suberin lamella is thought to carry out the additional function of restriction of apoplastic leakage of CO₂ from C₄ BS cells to the intercellular airspace, thus contributing to the maintenance of high CO₂ concentration and suppression of photorespiration (Hattersley and Browning, 1981). In maize, deposition of suberin in BS walls coincides with maturation of the last-formed xylem elements in the vein: the suberin lamella first appears at isolated sites within the BS cell walls and becomes continuous in each cell as the last xylem elements mature (Evert et al., 1996). Just before maturation, the suberin lamellae become thickened and polylamellate in regions of the BS/M cell wall with conspicuous aggregations of plasmodesmata. Positional regulation of suberin lamella deposition is presumably unaltered during leaf development in NADP-ME grasses, but, in PK type C₄ grasses, suberin lamellae are deposited in both the inner mestome sheath cell walls and in the outer bundle sheath layer (the C₄ BS cells) (Hattersley and Browning, 1981; Eastman et al., 1988). Thus the evolution of Kranz anatomy in PKC grasses requires novel spatial control of suberin lamella deposition and associated modification of the suberin lamella at the site of plasmodesmatal aggregations (Fig. 1C).

D. Plasmodesmata and Cell-Cell Communication in C₄ Development

Both spatial and temporal patterns of BS and M cell differentiation indicate that cell position determines cell fate and that cell-cell communication is essential for interpretation of position (Langdale and Nelson, 1991; Nelson and Langdale, 1992). For instance, ground tissue cells differentiate as C₄ M cells only when adjacent to BS cells, indicating that a BS-derived signal is required for determination of cell identity (Langdale et al., 1988b). In maize, the modified husk leaves that surround the female...
Plasmodesmata form cytoplasmic channels between adjacent cells and provide a potential route for the intercellular movement of signaling molecules. High densities of plasmodesmata are present at the BS–M cell interface in the mature leaves of C₄ plants and provide an extensive symplastic pathway for the diffusion of C₄ acids and pyruvate between BS and M cells (Botha and Evert, 1988; Robinson-Beers and Evert, 1991a; Evert et al., 1996). It is possible that this pathway also functions in the exchange of informational molecules that coordinate metabolism, as suggested for communication between the M cells and phloem companion cells that function in sieve tube loading in C₄ leaves (Kragler et al., 1998). Recent evidence indicates that such a pathway may also function in the regulation of developmental processes. Analysis of genetic mosaics has shown that a number of genes with important developmental roles act non-cell autonomously. For instance, mutations in the maize KNOTTED1 (Kn1) homeobox gene result in the formation of ectopic knots and ligule fragments on the surface of the maize leaf blade (Smith et al., 1992). These structures are derived from epidermal tissues, yet are formed in wildtype tissue when the middle mesophyll layer carries a mutant allele (Hake and Freeling, 1986). Thus internal layers must be able to communicate with surface tissues to induce the alteration in cell division planes that produces these structures. Microinjection experiments have shown that fluorescein-labeled KN1 protein can move symplastically between M cells, effectively mediating its own transport through plasmodesmata by increasing the size exclusion limit (Lucas et al., 1995).

Developmental changes in the plasmodesmata that interconnect vascular tissue, BS cells, and M cells may affect the potential pathway for the movement of macromolecular developmental signals. Primary plasmodesmata are formed during cytokinesis and thus provide cytoplasmic continuity among cells that share a cell lineage. During leaf development in C₄ NADP-ME type grasses, the cell lineage that gives rise to provascular tissue (and ultimately xylem, phloem and bundle sheath) is distinct from the surrounding ground meristem lineage that gives rise to mesophyll (Dengler et al., 1985). In addition, the layer at the vein periphery that gives rise to the bundle sheath layer forms a distinct lineage from inner provascular layers at early stages of vein development. Thus, initially, plasmodesmatal connections would provide only a limited pathway for the movement of signal molecules between BS and M cell precursors (Fig. 3B). The original patterns of primary plasmodesmatal connections can become highly modified during development, however, by the formation of secondary plasmodesmata (Fig. 3C-E); this modification essentially overrides the
potential limitation of cell lineage on development by establishing new pathways for cell–cell communication (Lucas, 1995; Kragler et al., 1998).

At present, there is only limited evidence for the formation of secondary plasmodesmata at the BS–M cell interface in C₄ plants. In developing leaves of the NADP-ME type grass *Arundinella hirta*, only a few isolated plasmodesmata are present in the shared BS–M cell wall at the provascular stage (Dengler et al., 1996), but as BS and M cells differentiate, new aggregations of plasmodesmata appear and the mean density of plasmodesmata remains constant, despite a many-fold increase in cell surface area. Detailed ultrastructural observations of plasmodesmata development in the C₄ NADP-ME type grasses maize and sugar cane did not detect secondary plasmodesmata formation, however, possibly because these studies focused on later developmental stages (Robinson-Beers and Evert, 1991a; Evert et al., 1996).

In maize, plasmodesmata at the BS–M cell interface mature shortly before sink to source transition when BS and M cells are fully differentiated. Thus, while there is clear evidence for a substantial pathway for cell–cell communication in almost-mature and mature leaves, this pathway appears to be limited at early stages of development when differences in BS and M cell structure and cell-specific expression of photosynthetic enzymes and their mRNAs begin to be expressed. Although the experiments showing that *KN1* protein effectively altered the size exclusion limit of plasmodesmata were conducted using mature tobacco leaves, *KN1* protein occurs in the outer layer of the maize shoot apical meristem where *KN1* mRNA is undetected, suggesting that macromolecules can move through the plasmodesmata of undifferentiated tissues (Jackson et al., 1994; Lucas et al., 1995). Therefore it is possible that a putative signal, required for the induction of the C₄ pattern of BS and M cell differentiation, moves between cell layers even at a stage when the interconnecting plasmodesmata are few and possibly immature.

While plasmodesmata may play an important role in cell–cell communication during development of C₄ photosynthetic tissues, there may be additional or alternative mechanisms involved in the interpretation of positional information. In common with other eukaryotic organisms, plants are known to possess receptor-like molecules with receptor kinase activity that are located in the plasma membrane (Walker, 1994). Plants possess a large family of receptor-like kinase genes, and each gene likely encodes a distinct protein kinase that may play a unique role in cellular signaling. For instance, mutant *erecta* (*er*) phenotypes in *Arabidopsis* indicate that this gene plays a role in the coordination of cell growth patterns during shoot development (Torii et al, 1996). *ERECTA* encodes a putative transmembrane receptor kinase with an extracellular ligand binding domain, indicating that it influences development through its role in intercellular signal transduction. Similarly, the *CRINKLY4* (*CR4*) gene encodes a receptor-like kinase and is required for normal epidermal cell patterning in the leaves of maize (Becraft et al., 1996). Genes with a similar function are likely involved in signaling between provascular tissue and surrounding cells early during C₄ leaf development (Fig. 3), but have not yet been identified from mutant screens.

V. Compartmentation of Photosynthesis

A. Genes Coding for C₄ Enzymes

The efficiency of C₄ photosynthesis is dependent on the strict compartmentation of enzyme activities responsible for the fixation and subsequent reduction of atmospheric CO₂. The compartmentation of C₄ cycle enzymes is due to cell-specific expression of their corresponding genes (Fig. 3E). In principle, this could be accomplished by a simple mechanism in which a factor specific to each cell type is produced during the differentiation of M and BS cells. Each factor would interact with a common cell-specific DNA sequence to induce and/or repress the expression of a set of genes in that cell type. A simple model for the evolution of genes coding for C₄ enzymes would involve gene duplications to create new copies of genes already coding for non-photosynthetic functions followed by the acquisition of cell-specific sequences as well as regulatory sequences for high-level, light-induced expression. However, analyses of gene structures and programs of expression has failed to find any evidence of a simple master switch mechanism; rather, the evidence points to a surprising diversity in gene structure, composition of gene families and mechanisms of regulation. We will limit our discussion to the most studied genes, especially those for which regulatory mechanisms can be inferred. Although gene names may differ between species and labs, we will use a single name for each gene or family to avoid confusion.
PEPCase is encoded by a gene family in all plants studied to date (Ku et al., 1996). The dicot genus *Flaveria* has been particularly useful in making evolutionary comparisons between genes because some species show full development of the C₄ pathway, others show uniform C₃ characteristics, while another group are C₃-C₄ intermediates, showing a range of characteristics from C₃-like to C₄-like. Hermans and Westhoff (1990, 1992) compared *Ppc* genes in *Flaveria trinervia*, with full development of C₄ characteristics, and *F. pringlei*, a C₃ species. Both species have similar numbers of genes which were divided into four subfamilies based on sequence relatedness. In *F. trinervia*, one member of the *PpcA* subfamily was shown to encode a C₄ isoform of PEPCase, whereas *PpcB* and *PpcC* genes encode non-photosynthetic forms (Ernst and Westhoff, 1997). The *F. trinervia* *PpcA1* gene is more similar to the *F. pringlei* *PpcA1* gene than it is to any other *F. trinervia* *Ppc* genes. *PpcA* genes from the two species were therefore defined as being orthologous even though their patterns of expression are strikingly different. In *F. pringlei*, *PpcA* genes are expressed at low levels in leaves, roots and stems while *PpcA* expression is high-level and M cell-specific in *F. trinervia*. The identification of orthologous genes helps to define those whose comparison will best show evolutionary relationships of gene structures and regulatory mechanisms. We will use the term ‘non-photosynthetic isoform’ to describe gene family members encoding isoforms not involved in C₄ photosynthesis, rather than C₃ isoform. The latter term can be confusing, sometimes referring to non-photosynthetic forms in C₄ plants and other times referring to orthologous forms in C₃ plants.

In maize the different isozymic forms of PEPCase are encoded by a small gene family of about five members (Harpster and Taylor, 1986; Hudspeth et al., 1986) while sorghum has only three genes (Lepiniec et al., 1993) and amaranth has 10–20 genes (Rydzik et al., 1996). In each case, a subfamily of one or more members encodes the C₄ isozymic form. Our current understanding of *Ppc* gene families is consistent with the evolution of genes encoding the C₄ isoform through gene duplications.

Another case of likely gene duplication has been found with the small gene family encoding NADP-ME in *Flaveria* species. Marshall et al. (1996) identified two genes coding for chloroplastic NADP-ME, one of which, *Me1*, was shown to encode the C₄ isoform located in BS cells of *F. bidentis*, a C₄ species. The *Me2* gene was shown to be expressed at low levels throughout the plant. *Me1* was expressed at low levels in C₃ species and its level of expression in C₃-C₄ intermediate species was correlated with the degree of C₄ characteristics in each species.

In contrast, PPDK has been shown to be encoded by a single gene in *Flaveria* species (Rosche and Westhoff, 1995). However, two transcripts result from this single *Pdk* gene. One codes for the mature form of the enzyme plus a chloroplast transit sequence. This 3.4 kb mRNA is present at high levels in M cells and at lower levels in stems. A second, shorter RNA codes for only the mature form of the enzyme and is presumed to be cytoplasmically localized. This mRNA is present only at very low levels and is found in roots and stems. Maize has a dual function *Pdk* gene that has a similar expression program to the *Flaveria* one, but it also has a second *Pdk* gene which codes only for the mature form (Glackin and Grula, 1990; Sheen, 1991; Matsuoka, 1995) and is expressed at very low levels.

**B. Genes for C₄ Photosynthesis Showing Differential Expression in C₄ Plants**

The restriction of the photosynthetic carbon reduction cycle (Calvin cycle) to BS cells in C₄ plants is due to altered expression of the genes encoding component enzymes. Most studies have focused on RuBPCase, whose LSu is encoded by the plastid gene *rbcL* and SSu by a small family of nuclear *RbcS* genes. Although the overall expression of genes from two different genomes is coordinated, recent work has shown that there are some differences within the *RbcS* gene family. Ewing et al. (1998) used gene specific probes to measure the expression of two *RbcS* genes in leafblades, where all cells are C₄, and husk leaves, where M cells distant from veins are C₃. Both genes were expressed at high levels in leaf blades but the *RbcS1* gene was preferentially expressed in husk leaves.

The glycine cleavage system of the photo-respiratory pathway is another example of enzymes active in C₃ plants that are restricted to BS cells in C₄ plants (Ohnishi and Kanai, 1983). The activity of GDCST is also BS-specific in C₃-C₄ intermediate species (Bauwe et al., 1987; Hylton et al., 1988). The complex has four subunits and is located in the mitochondrion. Each subunit is encoded by a single nuclear gene in *Flaveria* and the gene for the H subunit is differentially spliced in an organ preferential manner (Kopriva et al., 1995).

C₄ plants belonging to the NADP-ME group exhibit
dimorphic chloroplasts with those in the BS having very few granal regions and little to no PS II activity (Woo et al., 1970). The relative absence of PS II activity is not due to the complete absence of component polypeptides, rather three nuclear encoded extrinsic polypeptides of the oxygen evolving complex are deficient (Oswald et al., 1990). Chloroplast encoded PS II subunits were found to differ in quantity depending on the species and the stage of development. The light harvesting complex of PS II is also absent or present at very low levels in BS chloroplasts, caused by the absence of transcripts of the nuclear Cab gene family which codes for the chlorophyll a/b apoproteins (Broglie et al., 1984; Schuster et al., 1985).

VI. Gene Regulation Mechanisms

The differential expression of photosynthetic genes is a key component of the differentiation process of M and BS cells. To understand the mechanisms regulating differential expression, the first step has been to define the programs of expression of the genes described in the previous section. Most studies have been conducted in maize, amaranth and Flaveria species using RNA northern blots and protein western blots to measure mRNA and protein accumulation and in situ hybridization and immunolocalization techniques to identify expression patterns of mRNAs and proteins in tissue sections. These techniques have identified the steps of gene expression at which regulatory control is active and have provided hints as to the signals responsible for regulation.

The second experimental step has been to dissect the mechanisms of regulatory control. Genetic transformation techniques, both transient and stable, are being used to identify regulatory DNA sequences and the protein factors which interact with these sequences. These protein factors become important molecular probes for the third experimental step which is to bridge the gap between morphological differentiation at the organ and cell level and regulation of gene expression. Analysis of mutants affecting C₄ development and the growing field of signal transduction pathways also will contribute to this objective.

A. Temporal Regulation

Because significant amounts of C₄ enzymes, including RuBPCase, are not found prior to the differentiation of M and BS cells, temporal and spatial regulation of gene expression could be entirely controlled by the differentiation process. But detailed analyses have uncovered a more complex story, with differences found between genes and between species. Berry and colleagues used fluorescent in situ techniques to determine the patterns of accumulation of mRNAs and their protein products in amaranth. The mRNAs for PEPCase and PPDK were detected in apical meristems and leaf primordia, but the onset of accumulation of the two proteins was coincident with cellular differentiation in young leaves (Ramsperger et al., 1996). The mRNAs for both subunits of RuBPCase showed a similar pattern of accumulation in meristems and leaf primordia, but the LSu and SSu polypeptides were also detected in leaf primordia prior to cell differentiation. Both polypeptides became BS cell-specific in a basipetal pattern which correlated well with the transition of leaf metabolism from sink to source (Wang et al., 1993b). The α-subunit of NADP-ME, although located in BS cells, shows a pattern of accumulation similar to M cell-specific PEPCase and PPDK (Long and Berry, 1996). The α-subunit mRNA, however, is always found in BS cells and does not accumulate prior to cell differentiation.

In maize, the BS cell-specific mRNAs for SSu and NADP-ME were found to accumulate in leaf primordia prior to cell differentiation (Langdale et al., 1988a), while protein accumulation was concurrent with the development of Kranz anatomy. The accumulation of Ppc and Pdk mRNAs was coincident with cell differentiation (Langdale and Nelson, 1991; Nelson and Langdale, 1992).

Recent experiments with stably transformed Flaveria bidentis (C₄ species) provide additional evidence of C₄ gene activity prior to cell differentiation. The promoter of the Me1 gene, which codes for the BS cell-specific C₄ isoform of NADP-ME, directs gus gene expression at high levels in apical and axillary meristem regions (Marshall et al., 1997). Although it is possible that this promoter activity does not represent the activity of the intact gene, it is none the less interesting that the promoter does have a meristem active element. In contrast, the promoter from the Pdk gene did not direct gus gene expression in meristem regions (Rosche et al., 1998).

B. Hierarchy of Developmental Controls

The discoordinated accumulation of mRNAs and their proteins described in the previous section
indicates that the differential expression of \( \text{C}_4 \) genes is regulated at post-transcriptional as well as transcriptional levels. Although \( Ppc \) and \( Pdk \) mRNAs were detected in amaranth leaf primordia, they became cell-specific with the differentiation of M cells and greatly increased in abundance (Ramsperger et al., 1996). Transcriptional regulation could account for the M cell-specific increase in mRNA abundance and its disappearance from BS cells could be due to selective mRNA degradation or normal mRNA turnover in the absence of BS cell transcription. Of course, an increase in mRNA could also involve a regulated increase in its stability, not just its rate of synthesis. Regulation at the level of translation or protein turnover must be responsible for the delayed accumulation of PEPCase and PPDK polypeptides. Their initial accumulation occurs in cells destined to become M cells as judged by their position relative to developing veins in amaranth leaves (Ramsperger et al., 1996). With further growth and development both polypeptides become M cell-specific.

Both \( rbcL \) and \( RbcS \) mRNAs accumulate in amaranth meristems and leaf primordia (Ramsperger et al., 1996). The levels of both mRNAs are relatively high in young leaves and are not BS cell-specific until a later stage. Both SSu and LSu polypeptides were first detected in the ground meristem of leaf primordia and then became progressively localized in pre-BS cells even though their mRNAs were still present at high levels in pre-M cells. Berry and colleagues have described the first stages of \( rbcL \) and \( RbcS \) gene expression as a default \( \text{C}_4 \)-like pattern. The transition to the BS cell-specific \( \text{C}_4 \) pattern is correlated with the transition from sink to source metabolism as the \( \text{C}_4 \) pathway becomes functional in fully differentiated M and BS cells (Wang et al., 1993b).

Maize leaves grown in the dark exhibit a default \( \text{C}_4 \)-like pattern of gene expression. RuBPCase is found in both cell types and \( \text{C}_4 \) mRNAs and proteins are not detectable. Transferring etiolated leaves to the light suppresses RuBPCase expression in M cells and induces the accumulation of \( \text{C}_4 \) enzymes in their appropriate cell type (Sheen and Bogorad, 1987; Langdale et al., 1988b).

A number of strategies have been used to identify the \( \text{cis} \)-acting DNA sequences responsible for regulating the expression of \( \text{C}_4 \) genes. These include comparisons of DNA sequences in the flanking non-coding regions of closely related \( \text{C}_4 \) and non-\( \text{C}_4 \) genes and the identification of binding sites for nuclear proteins which are presumed to have regulatory function by virtue of their sequence-specific binding. The most powerful strategy is to identify key regulatory sequences by loss or gain of function when fused to a reporter gene and assayed in transformed cells or whole plants. A major difficulty with this approach has been the lack of a reliable and efficient transformation system for any \( \text{C}_4 \) plant. To overcome this limitation, reporter gene activity has been assayed in transiently transformed cells or organs of \( \text{C}_4 \) plants or in stably transformed \( \text{C}_4 \) plants. Both approaches have provided useful information and, in some cases, have fueled interesting speculation about the evolution of \( \text{C}_4 \) gene regulation. Recently, stable transformation systems have been developed for the \( \text{C}_4 \) species \textit{Flaveria bidentis} (Chitty et al., 1994) and the \( \text{C}_4-\text{C}_3 \) intermediate species \textit{F. pubescens} (Chu et al., 1997). These systems have proven to be efficient and reasonably rapid so that a small research laboratory is able to generate an adequate number of transformed plants to make progress in the identification of \( \text{cis} \)-acting DNA sequences. Transformation of maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), sugarcane (Bower and Birch, 1992), and sorghum (Casas et al., 1993) have been reported but these systems have not so far provided a useful level of efficiency, speed, or accessibility. However, recent improvements in maize and sugarcane transformation may change the picture (Ishida et al., 1996).

Sheen and coworkers developed a transient expression system using protoplasts isolated from maize tissues which include dark- and light-grown leaves. Although it must be kept in mind that the process of making protoplasts may induce cellular dedifferentiation, Sheen’s method has been shown to retain cell specialization and to reflect light-induced changes in gene expression (Sheen, 1990, 1991). Leaf protoplasts are primarily M cells. This approach was used to identify the promoter region of the complex, dual function maize \( Pdk \) gene (Sheen, 1991) and to identify sequences from the 5′ regions of the \( \text{C}_4 \) \( Ppc \) gene (Schäffner and Sheen, 1992) and the \( RbcS \) genes (Schäffner and Sheen, 1991) which are involved in high level leaf expression. Positive and negative acting promoter elements responsible for regulation of transcriptional activity were identified. In addition, evidence for post-transcriptional regulation of maize \( RbcS \) genes was also presented (Sheen, 1990). \( RbcS \) promoter constructs were shown to be active in M cell protoplasts.
Matsuoka and Numazawa (1991) performed a similar analysis of the maize Pdk gene using biolistic delivery of constructs to maize leaves to measure transient reporter gene activity. They identified an element that conferred high level M cell expression and showed specific binding of a nuclear protein, presumably, a trans-acting regulatory factor. While these transient expression assays have been useful in identifying DNA sequences controlling high-level, leaf-specific expression, the analysis of sequences controlling cell specificity is more readily accomplished in stably transformed plants. As was expected, transformation of C₄ gene constructs into C₃ plants has helped in the analysis of light regulation and high-level expression in leaves. But this approach also has provided some insights into the mechanisms of cell-specific gene regulation and their evolutionary origins.

Maize Pdk, Ppc and RbcS genes have been transformed into rice, which has the advantage of a well differentiated bundle sheath as is true of all grasses. The Pdk and Ppc promoters conferred high-level reporter gene expression preferentially in M cells (Matsuoka et al., 1993, 1994). The authors concluded that rice, although a C₄ plant, has trans-acting factors capable of recognizing M cell-specific sequences in both C₄ genes. Nuclear proteins with similar binding properties as found in maize were isolated from rice for both promoters (Matsuoka et al., 1993, 1994). The major change in the evolution of both C₄ genes must have been the acquisition of cis-acting sequences controlling M cell-specific expression. These results, and those from the maize leaf protoplast experiments (Sheen, 1991; Schäffner and Sheen, 1992), suggest that M cell-specificity is regulated primarily by differential transcription of C₄ Ppc genes. However, a maize RbcS promoter also directed high-level expression in M cells in rice (Matsuoka et al., 1994). The authors conclude that the trans-acting factors that suppress RbcS expression in C₄ plants must be absent from rice where RbcS genes are expressed at high levels in M cells.

Stockhaus et al. (1994) compared the activities of two Ppc promoters in tobacco. One promoter was from the PpcA1 gene of Flaveria trinervia, a C₄ species, which encodes the C₄ isoform, and the other was from the orthologous PpcA1 gene of F. pringlei, a C₃ species. The F. trinervia promoter directed high-level, leaf-specific expression in tobacco, primarily in the palisade mesophyll. The F. pringlei promoter directed low-level expression in leaves, stems and roots, a pattern similar to that of the PpcA1 gene in F. pringlei. These authors also conclude that new cis-acting sequences played a major role in the evolutionary origin of the F. trinervia PpcA1 gene and that trans-acting factors necessary for high-level, M cell expression are present in C₃ plants. Both promoters were recently transformed into F. bidentis, a C₄ species closely related to F. trinervia (Stockhaus et al., 1997). The F. trinervia PpcA1 promoter directed high-level M cell-specific reporter gene expression whereas the promoter from F. pringlei was active at a low level in leaves and stems but not roots. These results confirmed the importance of cis-acting sequences in the C₄ gene and provide clear evidence that transcriptional regulation may be the primary level of M cell-specific control of the C₄ Ppc gene in Flaveria.

Transcriptional regulation also appears to be responsible for M cell-specific expression of the F. trinervia Pdk gene (Rosche et al., 1998). Sequences flanking the coding region for the chloroplast form of the enzyme were found to direct high-level M cell expression in transgenic F. bidentis plants. Sequences from this same region were also shown to direct lower-level expression in BS cells and in stems and roots, suggesting that M-specific cis-acting sequences were added to a non-photosynthetic gene without disrupting the original expression program of that gene. Photosynthetic and non-photosynthetic functions are thereby provided by one gene without duplication.

A more complex story has emerged from studies of the F. bidentis Me1 gene, which codes for the C₄ isoform of NADP-ME. Sequences from the 5′ end of the gene were found to direct only very low levels of gus gene expression in leaves. By using cell separation techniques and a very sensitive fluorescence assay for GUS enzyme activity, Marshall et al. (1997) were able to show that this low level of GUS was primarily in BS cells. When sequences from the 3′ end were added to the gus gene construct with Me1-5′ sequences, high-level GUS activity was found preferentially in BS cells. In contrast with other C₄ gene promoters, none of the Me1 constructs showed any significant expression in transformed tobacco. Marshall et al. (1997) concluded that sequences at the 5′ end of the gene controlled BS specificity and sequences at the 3′ end are responsible for high level expression, but the interaction of 5′ and 3′ sequences were dependent upon a trans-acting factor only present in C₃ plants. Experiments are in progress to
determine if the Me1-3’ sequences act as a transcriptional enhancer or if they affect post-transcriptional processes. Because reporter gene expression was detected in M cells, the authors concluded that additional, post-transcriptional mechanisms were required to make NADP-ME accumulation strictly BS cell-specific. The 5’ region of the Me1 gene was also shown to direct high-level expression in the meristematic regions of the shoot apex and axillary buds, a pattern similar to that described for Pdk and Ppc mRNA accumulation in amaranth (Ramsperger et al., 1996). High-level GUS activity was detected in stems which was primarily localized in cells of the vascular cambium and xylem parenchyma. These cells showed the highest chloroplast numbers but no evidence of C4-type cellular differentiation, suggesting that promoter activity can be induced by a photosynthetic developmental program without Kranz-type differentiation.

Sequences at the 3’ end of the maize RbcS gene are also involved in cell specificity. Viret et al. (1994) introduced reporter gene constructs into maize leaves by microprojectile bombardment and showed that 3’ sequences were responsible for suppressing RbcS promoter activity in M cells but had little effect on quantitative levels. It is not known whether the RbcS 3’ sequences affect transcription or a post-transcriptional process.

The glycine cleavage system is restricted to BS cells in C4 and C3-C4 intermediate plants. The promoter activity of the gene encoding the T protein, GDClST, has been measured in stably transformed F. pubescens, a C3-C4 intermediate species, and in tobacco (H. Bauwe, personal communication). Sequences from the 5’ end of the gene confer BS cell-specific GUS expression in F. pubescens. This same construct directs GUS expression in a BS-like pattern in tobacco; GUS is detected in cells adjacent to veins but is relatively absent in mesophyll cells that are more than one or two cells distant from a vein. These authors conclude that trans-acting factors responsible for BS cell-specific expression are present in the bundle sheath of C4 plants and that the evolutionary acquisition of BS-specific cis-acting sequences has been primarily responsible for the changed localization of GDC proteins in C4 and C3-C4 intermediate species. These data and conclusions contrast with those of Marshall et al. (1997) on the Me1 gene, whose expression appears to be dependent on a trans-acting factor only present in the C4 plant.

The compartmentalized expression of the genes coding for C4 enzymes is accomplished by complex mechanisms which activate high level expression of each gene in its appropriate cell type and, in some cases, repress the expression of that gene in the other cell type (Fig. 3E). Cell-specific repression may be particularly important for genes encoding components of C4 photosynthesis whose expression must be redirected in C4 plants.

C. Role of Environmental Cues

Light is the principle environmental signal that influences plant development. A great deal of current experimental effort is devoted to understanding light perception and the transduction of light signals which induces or represses gene expression and alters programs of development (Chory, 1993; von Arnim and Deng, 1996). Light affects the expression of C4 genes in at least two ways. Light has been shown to act directly on the transcription of a number of nuclear genes, the best studied cases being RbcS and Cab in C4 plants (Tobin and Silverthorne, 1985; Thompson and White, 1991). But light also has an indirect effect on gene expression by first activating further development of leaf cells and of the chloroplasts in them. In this case, the role of light may be to remove a developmental block so that leaf cell development can proceed far enough for the gene to become competent to respond to direct light activation. Just as C4 genes have different programs of regulation, so are their responses to light different. When constructs with the maize Pdk or Ppc promoters were electroporated into etiolated maize leaf protoplasts, light was able to directly activate the Pdk (Sheen, 1991) but not the Ppc promoter (Schaffner and Sheen, 1992). The Ppc promoter was activated in protoplasts from greening leaves.

Light also has a major role in regulating cell-specific gene expression. Langdale and Nelson (1991) showed that RbcS and rbcL transcripts accumulated in both cell types in etiolated maize leaves, but at relatively low levels. Light increased the levels of both mRNAs in BS cells and caused them to decrease in M cells. Langdale and Nelson (1991) have concluded that light was responsible for the induction of a regulatory signal emanating from veins, which induces C4 cell type differentiation. As described previously, a significant aspect of C4 cell differentiation is the induction of cell-specific expression of C4 genes.
Although this chapter is focused on the assembly of the C\textsubscript{4} pathway during leaf development, it must be remembered that light cues can affect gene expression at all stages. The products of photosynthesis affect the expression of genes coding for components of the photosynthetic apparatus through a sensing mechanism involving simple sugars (Koch, 1996; Jang and Sheen, 1997). Environmental variables such as light will have a major effect on the rates of photosynthesis and sugar production. Light cues also can induce leaf senescence which in turn will reduce gene expression.

**D. Possible Mechanisms of Hierarchical Control**

Although differences are seen in the patterns of expression among genes coding for components of the C\textsubscript{4} pathway and in the apparent mechanisms controlling their expression, there are some common principles governing the regulation of these genes. One is that expression of C\textsubscript{4} genes is controlled not only by the differentiation of BS and M cells but also by the developmental program of the leaf. In some cases this organ-specific regulation is evident as gene transcription commences in the leaf primordium or in leaf parenchyma cells prior to cell differentiation. The fact that promoters of some C\textsubscript{4} genes also show leaf-specific expression in transgenic C\textsubscript{3} plants indicates that these promoters respond to developmental signals controlling leaf development. Another indication of regulation at the organ level in C\textsubscript{4} plants is the observation that, prior to M and BS cell differentiation, genes, such as \textit{RbcS}, show a ‘default’ C\textsubscript{4} pattern of expression in leaf parenchyma cells with transcripts accumulating in pre-M cells as well as pre-BS cells.

High-level expression of photosynthesis genes occurs only in those cells where chloroplasts develop and replicate to high numbers. In a C\textsubscript{4} plant, positional signals in the developing leaf are thought to be responsible for activating plastid differentiation and replication and for inducing the expression of photosynthesis genes in both genomes (Taylor, 1989). That early transcriptional activity of C\textsubscript{4} genes occurs primarily in regions giving rise to M and BS cells suggests that this same positional signaling also regulates the expression of C\textsubscript{4} genes.

The developmental status of the chloroplast has been shown to influence the expression of several nuclear photosynthesis genes through an hypothesized chloroplast signal (Oelmuller, 1989; Taylor, 1989). The functional role of this signal is not clear nor is its mechanism of action. It has been proposed to provide feedback-like information required for high-level expression of photosynthesis genes in the nucleus (Taylor, 1989). Whether this chloroplast signal also affects the expression of C\textsubscript{4} genes is not clear. Burgess and Taylor (1987) provided evidence that maize \textit{Pdk} gene expression could be at least partially controlled by the chloroplast signal, but expression of the C\textsubscript{4} \textit{Ppc} gene, which encodes a cytoplasmic enzyme, was not. However, the maize mutation, \textit{bsd3}, which blocks chloroplast development at a very early stage in both cell types, affects only the accumulation of C\textsubscript{4} proteins, not C\textsubscript{4} pathway enzymes (J. A. Langdale, personal communication). A correlation between C\textsubscript{4} gene expression and the developmental status and/or photosynthetic competence of the chloroplast can also be seen in \textit{Amaranthus tricolor}. Some leaves have non-photosynthetic red and yellow sectors where the synthesis of C\textsubscript{4} enzymes is significantly reduced (McCormac et al., 1997). Yellow sectors have normal Kranz anatomy and vein patterns, however, there is a loss of cell-specificity of \textit{rbcL} and \textit{RbcS} transcripts, along with decreased rates of transcription of several plastid genes. It is possible that in some of these examples the developmental status of the chloroplast is merely one indicator of the developmental status of the cell, which is the primary regulator of gene expression.

As illustrated in Fig. 4, we divide the hierarchy of gene regulation in C\textsubscript{4} leaves into two major levels. The first level involves the establishment of the potential for high-level gene expression in those cells in the leaf primordium that are determined to become photosynthetically active. This determination is accomplished by positional signals yet to be identified. It is possible that the same positional signals that activate chloroplast development and replication also establish the potential for high-level expression. In some cases the potential is evident in the accumulation of transcripts in the leaf primordium prior to differentiation. In other cases the potential is not realized until cell differentiation occurs and high level gene expression occurs in a cell-specific fashion. This level of regulation will result in the C\textsubscript{4} ‘default’ pattern of expression that is seen for some C\textsubscript{4} genes in the early stages of leaf development. It probably uses the same positional signals as in the development of a C\textsubscript{3} leaf, the only difference being the establishment of high expression potential in pre-BS
cells of \( \text{C}_4 \) leaves. It should be emphasized that \(' \text{C}_4 \) default' refers only to the pattern of gene expression and does not mean that \( \text{C}_3 \) photosynthesis occurs prior to the differentiation of M and BS cells.

The appearance of \( \text{RbcS} \) and \( \text{rbcL} \) transcripts in the apical meristem of amaranth (Ramsperger et al., 1996) indicates that this first level of regulation will turn out to be more complex than the simple version presented here. Just as cell fate is determined by progressive interpretation of positional signals, the activation of the potential for high-level photosynthetic gene expression most likely will occur in a progressive fashion.

The second level of regulation in leaves of \( \text{C}_4 \) plants involves the differentiation of M and BS cells (Fig. 4). As discussed above, Langdale and Nelson (1991) have presented evidence in maize for a positional signal arising in veins that promotes the differentiation of the adjacent ring of parenchyma cells into BS and the cells adjacent to them into M. This differentiation process has two consequences for gene expression. One is the full activation of
high-level expression of photosynthesis genes and the other is the restriction of the expression of some genes to one cell type. Even with genes whose transcripts accumulate prior to the differentiation of Kranz anatomy, high-level expression is only evident in differentiated cells.

It is not clear if the same developmental signal is responsible for activating high-level expression and restricting expression to one cell type. Histochemical analyses of mRNA and protein accumulation suggest that the two processes occur simultaneously. However, regulation at multiple levels is involved in controlling the expression of some genes, indicating that mechanisms may be complex. Cell-specific gene expression is, in some cases, due to a combination of activation in one cell type and repression in the other. The evidence to date also indicates that each \( \text{C}_4 \) gene uses its own regulatory mechanism to achieve cell specificity. There is no obvious similarity between cis-acting DNA sequences within promoters nor is there any obvious similarity in the protein factors binding to these sequences. The recently discovered Dof1 factor in maize has been shown to transactivate expression of only the \( \text{C}_4 \) Ppc promoter and have no effect on the activities of \( \text{Pdk}, \text{RbcS} \) or \( \text{Cab} \) promoters (Yanagisawa and Sheen, 1998). Even if all M cell-specific genes respond to the same M cell signal, this signal seems to activate different mechanisms which in turn activate or repress the expression of \( \text{C}_4 \) genes.

Leaf-specific expression of \( \text{C}_4 \) transgenes in \( \text{C}_3 \) plants could be said to provide additional evidence for the first level of regulation by the developmental program of the leaf. Promoters from \( \text{C}_4 \) genes are recognized by transcription factors in the \( \text{C}_3 \) leaf so that expression is activated in photosynthetic cells. However, the fact that \( \text{C}_4 \) promoter activity shows some cell preference in \( \text{C}_3 \) leaves has also led to the conclusion that there are cell positional signals in a \( \text{C}_3 \) leaf which can interact with the cell-specific cis-acting sequences of the \( \text{C}_4 \) transgene. Maize \( \text{Ppc} \) and \( \text{Pdk} \) promoters are expressed in M but not BS cells of rice (Matsuoka et al., 1993, 1994) as is the \( \text{RbcS} \) promoter. It must be kept in mind that the rice BS cells do not show full development of photosynthetic characteristics, such as large numbers of chloroplasts. Therefore, the lack of expression of \( \text{C}_4 \) promoters in rice bundle sheath cells could be due to the lack of full activation of photosynthetic gene expression.

What are the signals responsible for developmental regulation of \( \text{C}_4 \) genes? Several experimental approaches are providing insights into these regulatory signals. One uses reporter gene fusions to define the functional cis-acting sequences of promoters. Proteins binding to these sequences are then good candidates for some components of the regulatory hierarchy. Once gene clones are isolated for these DNA binding proteins, their function can be tested in transgenic plants. Although this approach is extremely laborious, some progress is being made. Yanagisawa and Sheen (1998) exploited the maize protoplast transient expression system to show that a previously identified DNA binding protein, Dof1, activates transcription of the \( \text{C}_4 \) Ppc promoter but not other \( \text{C}_4 \) promoters. They were also able to show that although Dof1 was present in roots and stems, a related protein, Dof2, was responsible for repressing transcription in roots and stems. Furthermore, Dof1 activity was shown to be light dependent; binding to the Ppc promoter was significantly different in greening compared to etiolated protoplasts. The protoplast system may prove to be an efficient way of identifying nuclear proteins with regulatory activities.

An understanding of patterns of accumulation of these transcriptional regulators should tell us much more about mechanisms of developmental control. Because each \( \text{C}_4 \) gene appears to be controlled by a different set of DNA binding proteins it has been difficult to find common regulatory signals by examining the promoters of genes expressed in one or the other cell type. But studies of the expression programs of the genes coding for the regulatory proteins may reveal common control mechanisms. Similar approaches can be used to identify the regulators of post-transcriptional processes which are particularly important components of regulation at the second stage in the developmental hierarchy.

Another approach in the study of developmental regulation is to identify mutations that disrupt \( \text{C}_4 \) differentiation. A difficulty with this approach is the lack of an obvious phenotype. Langdale and colleagues screened pale-green maize mutants for those with alterations in amounts of \( \text{C}_4 \) enzymes or with cell-specific chloroplast defects. Although most mutants showed defects in both cell types a few have been identified that are specific to BS cells. These bundle sheath defective mutants appear to be defective in the differentiation of BS cells after the delineation of M and BS cells has occurred. Characterization of mutant phenotypes and, in one case, the isolation of the gene involved, has provided some insights into the control of Kranz differentiation and the relationships between the \( \text{C}_3 \) default level of cell
development and C₄ differentiation.

The bsd1 mutation causes aberrant development of BS chloroplasts and a major reduction in BS-specific C₄ enzymes (Langdale and Kidner, 1994). However, in dark-grown maize leaves bsd1 affects the accumulation of photosynthetic enzymes in both cell types. The authors propose that the Bsd1 gene acts in early stages of leaf cell development at the C₄ default level but its activity becomes restricted to BS cells once C₄ differentiation occurs. The Bsd1 gene has recently been isolated and shown to encode a nuclear-localized protein with similarities to transcription factors (Hall et al., 1998). Bsd1 gene expression is primarily restricted to BS cells. Another maize mutation, bsd2, also disrupts BS chloroplast development and blocks the accumulation of RuBPCase (Roth et al., 1996). However, both RbcS and rbcL transcripts are present in mutant leaves and rbcL transcripts accumulate ectopically in M chloroplasts as well as in the BS. Roth et al. (1996) speculate that the Bsd2 gene is active in both cell types where its primary function is to control the accumulation of rbcL mRNA and its translational activity. The defect in BS chloroplasts appears to be pleiotropic, due to the loss of photosynthetic activity.

A third mutation, bsd3, affects chloroplast biogenesis in both cell types, but C₄ enzymes accumulate normally (J. A. Langdale, personal communication). However, the C₄ proteins, Cab and RuBPCase, are present in reduced amounts due to reduced levels of RbcL and Cab mRNAs. Because the mutant blocks chloroplast biogenesis at a very early stage, Langdale and colleagues (J. A. Langdale, personal communication) speculate that the early block in chloroplast development interrupts the signaling between chloroplast and nucleus necessary for normal Cab gene expression, perhaps disrupting the hypothetical chloroplast signal (Oelmuller, 1989; Taylor, 1989).

The bundle sheath defective mutations have provided unique insights into genes that control or facilitate the full development of photosynthetic capacity in BS cells and their interactions with the C₄ differentiation program. These mutations show that many genes function at all stages of leaf cell development, but the C₄ differentiation program may restrict their activity to one cell type. Gene function may also change, as is the case with Bsd2 which prevents rbcL accumulation in M cells and promotes its accumulation and translation in BS cells.

To date mutations have not been identified in genes controlling C₄ differentiation. Perhaps these mutations would have more deleterious phenotypes and their identification will require screening for different phenotypes than previously employed. It is also noteworthy that no mesophyll defective mutations have been identified by Langdale and colleagues despite analyzing more than 150 pale-green and yellow maize mutants (J. A. Langdale, personal communication). A mesophyll defective mutation was created using antisense technology in transgenic Flaveria bidentis: plants showing major reductions in the level of PPDK were severely retarded in growth and extremely sensitive to photoinhibition even when grown heterotrophically (Furbank et al., 1997), indicating that screening under the appropriate conditions may be necessary.

VII. Future Directions and Model Experimental Systems

One of the most consistent and striking features of C₄ leaf development is the alteration of vein pattern, yielding the short interveinal distances characteristic of mature C₄ leaves. In one sense, the developmental phenomenon is comparable to a heterochronic shift where one process is prolonged in relation to another; in this case the successive formation of minor veins is extended in relation to leaf expansion, so that mature vein spacing is altered. It is possible that genes involved in other heterochronic shifts such as those controlling the transition from juvenile to adult leaves in maize and Arabidopsis (Moose and Sisco, 1996; Telfer et al., 1997) might participate in the developmental regulation of this important early step in C₄ leaf development. Alternatively, and perhaps more likely, genes regulating the spacing of elements in a repeated pattern, such as stomata or trichomes on the leaf surface (Yang and Sack, 1995; Larkin et al., 1996), might also function in vein spacing. Regardless of the actual mechanism involved in C₄ vein spacing and its parallels in other developmental processes, identification of genes regulating vein spacing through mutagenesis screens will be an essential first step.

Gene expression resulting in both C₄ tissue pattern and biochemical compartmentation appears to be regulated by the interpretation of positional information. The organization of tissues around veins involves oriented planes of cell division and cell enlargement in bundle sheath and mesophyll
precursors, while maturation of BS and M cells requires differential patterns of chloroplast (and mitochondrion) replication and development, of organelle placement within the cell, of cell wall modification, and of photosynthetic gene expression. The spatial relationships between veins, BS and M cells, as well as the temporal sequence of developmental events, indicates that a vein-derived signal may be crucial for these developmental events. Currently, very little is understood about how such signals might be sent and perceived, although clonal analysis of genetic mosaics of mutant and wildtype tissue sectors clearly indicates that gene expression in one tissue layer may influence developmental pattern in a quite different layer (Hake and Freeling, 1986; Fowler and Freeling, 1996). Phenotypic analysis of mutants at the rough sheath1, hairy sheath frayed1, Knotted1, and four Liguleless loci indicate that these genes function in the interpretation of positional information by leaf cells (Fowler and Freeling, 1996). A clearer understanding of genes such as these that have major morphological effects when mutated might also shed light on the interpretation of positional information at a more subtle anatomical level.

Other approaches will also be important for dissecting C_4 developmental pathways. Genes such as ERECTA in Arabidopsis and CRINKLY4 in maize indicate that receptor-like kinases are involved in signal transduction pathways required for the proper formation of cell pattern (Torii et al., 1996; Becraft et al., 1996). Similar genes may be involved in communication between vein, bundle sheath and mesophyll tissues in C_4 leaves. Parallel pathways might involve the symplastic movement of informational macromolecules through modified plasmodesmata as demonstrated for the Kn1 protein in tobacco mesophyll cells (Lucas et al., 1995). Solving the mystery of cell-cell signalling during C_4 leaf development will require a combination of mutant gene identification, understanding the modes of gene action and regulation, and investigating the consequences of mutant gene defects at the level of cell biology. As an example of the approach that needs to be taken, mutants in the KNOLLE gene of Arabidopsis disrupt cell pattern within tissues, but not overall tissue pattern. KNOLLE encodes for a syntaxin-like protein and affects plane of cell plate formation (Lukowitz et al., 1996). Genes with functions similar to KNOLLE must act in response to signals that specify tissue position during C_4 leaf development.

Maize will likely play an important role in future research as a C_4 model system, especially if maize transformation efficiency improves to the point that it becomes accessible to academic research laboratories. Flaveria bidentis already fulfils the transformation role, but does not have much possibility for genetic analysis. Transformation approaches will be crucial to demonstrate the function of proteins potentially involved in generating and interpreting positional signals in C_4 leaf development, especially for candidate proteins identified in other plants, such as Arabidopsis. But the research community must not lose sight of the extensive developmental diversity among C_4 plants and become overly focused on one or two model systems. We must know enough about morphogenesis and developmental gene regulation in other C_4 plants to be confident of the generalizations from our model systems.

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Chapter 21

The Physiological Ecology of $C_4$ Photosynthesis

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C₄ photosynthesis is an evolutionary syndrome that concentrates CO₂ around Rubisco and in so doing reduces photorespiratory inhibition of photosynthesis to negligible levels. It is not a single pathway, but a syndrome of functionally similar modifications that utilize phosphoenolpyruvate carboxylation in mesophyll cells, and transport of four-carbon acids to an enlarged bundle sheath tissue where Rubisco is localized. At least 14 distinct types of C₄ photosynthesis have been recognized, reflecting the use of one of three decarboxylating enzymes and one to two cell layers around the periphery of the vascular bundle. Despite substantial variation in how C₄ plants accomplish CO₂ concentration, the net effect on photosynthesis is similar in all forms. Relative to C₃ plants, C₄ plants have enhanced photosynthesis at CO₂ levels below the current atmospheric level of 360 μmol mol⁻¹. Increases in temperature above 25 °C favor C₄ relative to C₃ photosynthesis because photorespiration increases in C₃ species as temperatures rise while in C₄ species it remains minimal. Thus, in the current atmosphere, C₄ species have higher temperature optima relative to C₃ species of similar life form and higher CO₂ assimilation capacity at the temperature optimum. Because rising CO₂ inhibits photorespiration, the photosynthetic advantage of C₄ plants at warmer temperature is reduced or eliminated in high CO₂ conditions. C₄ plants have higher water and nitrogen use efficiencies than C₃ plants. This occurs because the capacity of C₄ systems to saturate Rubisco with CO₂ at low atmospheric CO₂ levels enables C₄ species to operate at lower stomatal conductances and Rubisco contents than C₃ species of equivalent CO₂ assimilation capacity. However, light use efficiency (quantum yield) differences between C₃ and C₄ depend on temperature. At current atmospheric CO₂ levels, C₃ species have higher quantum yields than C₄ plants below about 25 °C but lower above 30 °C. In C₄ plants, quantum yields do not change with temperature and CO₂ variation as they do in C₃ species, but do show differences between the biochemical subtypes. Species using NADP-malic enzyme generally have higher quantum yields than NAD-malic enzyme types for reasons that remain unclear. Differences in CO₂ leak rates had been suggested as a possible cause but recent permeability estimates do not show consistent variation between subtypes.

Ecologically, the C₄ pathway promotes fitness in warm environments receiving greater than approximately 30% of full sunlight intensities. C₄ species are generally absent in environments where average growing season temperatures are less than 15 to 18 °C, yet potentially dominate environments where the growing seasons are on average warmer than 22 °C. In warm climates, the dominance of C₄ species is largely dependent upon the availability of summer precipitation and conditions that inhibit establishment and dominance of woody vegetation. Where soil conditions (arid or infertile) and ecological disturbances such as fire restrict woody vegetation, C₃ species are abundant if not dominant. In general, however, moisture, salinity or low soil fertility have a subordinate role over abundance in that the dominant factors of temperature and light must be favorable or else C₄ species will not be competitive. Where intermediate temperatures favor neither photosynthetic pathway, however, drought, high salinity and nitrogen deficiency are important secondary controls, and appear to promote C₄ success in environments that otherwise would support C₃ dominance.

In the future, the distribution and abundance of C₄ species may become restricted because C₃ species generally respond more to rising atmospheric CO₂ than C₄ plants. Paleocology studies indicate that the direct consequences of rising CO₂ will be most important in the tropics, with woodland ecosystems potentially spreading into C₄ grasslands. In temperate zones, paleocological studies indicate that rising CO₂ and temperature could offset each other. If this occurs, other key ecological controls could become paramount; in particular, changes in the seasonality of precipitation could be important. Everywhere, human land use practices will have to be considered, given that people can radically alter vegetation characteristics depending upon their needs and desires.
Chapter 21  The Physiological Ecology of C₄ Photosynthesis

I. Introduction

The discovery of the C₄ photosynthetic pathway in the mid-1960s is one of the more important developments in plant biology. In addition to improving understanding of photosynthetic physiology, the C₄ discovery provided mechanistic insights into many ecological and evolutionary phenomena. This understanding also has major economic significance because photosynthetic pathway is a determinant of where and when crops species can be successfully cultivated. C₄ crops and pasture grasses perform well in hot, tropical environments, yet are unproductive in cool temperate climates unless they have been bred to complete their lifecycle during short growing seasons, as is the case for maize (Zea mays, C₄) grown in Europe. C₄ species are important converters of solar energy into biologically-useful forms required by humanity to meet its food and animal protein needs. Presently, about one-third of world grain production is C₄ and half of the world’s meat production is based on C₄ grains and forages (estimated from statistics in FAO, 1990). The contribution of C₄ biomass to the world’s food supply will increase in coming decades because human population growth and the consequent development of new agricultural land is concentrated in low latitudes where C₄ species perform well.

While the ecological and economic significance of the C₄ pathway has long been recognized, understanding of the evolutionary significance of C₄ photosynthesis is a relatively recent development. C₄ photosynthesis independently evolved at least 30 times in widely diverse lines of monocots and dicots, and is now present in about 8000 species from 18 families of flowering plants (about 4500 grass species, 1500 sedges, and 2000 dicots; Sage et al., 1999a). The evidence points to a recent origin in geologic time, between 15 and 30 million years ago (Cerling, 1999; Kellogg, 1999), with a rise to dominance on open landscapes of the tropics and subtropics between 5 and 8 million years ago (Cerling et al., 1997). Currently, C₄ photosynthesis is common, if not dominant, in biomes that cover about 40% of the surface of the Earth. In addition to their current

significance, C₄ plants have played an important role in human affairs throughout our history on this planet, affecting the development of both ancient and modern civilizations (van der Merwe and Tschauner, 1999). Our existence as a species may in part depend upon the success of the C₄ syndrome. The spread of the C₄-dominated grasslands and savannas in the last 5 to 8 million years contributed to the opening of wooded landscapes in East-Africa where early humans first evolved (Cerling et al., 1997). The environmental challenges of this open landscape were substantially different than in wooded ecosystems, and may have selected for many distinguishing characteristics of our species, such as upright stature, sophisticated weaponry, and complex social organization (Stanley, 1995).

With the growing awareness of the importance of C₄ plants to natural and human ecosystems has come an increased need for an up-to-date summary of the physiological ecology of the C₄ syndrome. No longer is an understanding of C₄ physiology and ecology solely the domain of plant scientists; it is also important for land managers, conservationists, anthropologists, geologists, and atmospheric scientists, to name a few. In this chapter, we review the physiological, ecological and geographic significance of the C₄ syndrome in a format that scales from biochemistry to landscape ecology. We also discuss the relationship between global change and the dynamics of C₃/C₄ interactions, providing some educated speculation about how C₄ plants may fare in the human-dominated world of the future.

II. Physiological Considerations

The physiological rational for the existence of C₄ plants arises from a fundamental flaw in the reaction mechanism of the primary carboxylating enzyme in all plants, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

A. Oxygen Inhibition of Rubisco Activity

Rubisco is a dual-function enzyme, catalyzing either the carboxylation or oxygenation of RuBP (Andrews and Lorimer, 1987). A product of RuBP oxygenation is phosphoglycolate (PG), a two-carbon compound that has no apparent value to plants and whose accumulation is toxic. Plants metabolize PG to PGA using ATP and reducing power, but in the process,
previously fixed carbon is lost as CO₂. Photorespiration (RuBP oxygenation and the metabolism of PG to PGA) inhibits photosynthesis by 1) competing for Rubisco active sites, 2) consuming RuBP, ATP and reducing power, and 3) releasing previously fixed (Sharkey, 1985, 1988). The degree to which photorespiration inhibits photosynthesis depends upon temperature and the stromal concentration of and (Andrews and Lorimer, 1987). Rising temperature substantially enhances the rate of photorespiration by reducing the specificity of Rubisco for relative to and by reducing the solubility of to a greater degree than it reduces the solubility for (Fig. 1A). At the current atmospheric CO₂ level, photorespiration is relatively modest below 15 °C; above 35 °C, photorespiration is highly inhibitory in all C₃ plants, reducing photosynthesis by over 30% (Fig. 1B).

Because of its potential to inhibit photosynthesis, there has been considerable discussion of the adaptive significance of photorespiration. No direct adaptive role has been identified, although indirectly photorespiration helps dissipate excess light energy (Takeba and Kozaki, 1998). A widely accepted view is that RuBP oxygenation is an unavoidable side reaction of the carboxylation mechanism and thus likely has no adaptive value. Its persistence through evolutionary time is a function of the critical importance of Rubisco to photosynthetic organisms and the presence of high levels of atmospheric CO₂ through most of earth’s history. Rubisco first evolved some 3 to 3.5 billion years ago in a setting where photorespiration was not possible because the atmosphere was highly enriched in CO₂ and lacking free O₂ (Hayes, 1994; Fig. 2A). Atmospheric conditions remained highly unfavorable for photorespiration for over three billion years, during which time an elaborate photosynthetic biochemistry centered on RuBP carboxylation evolved and became so successful that all photosynthetic life now employs the Rubisco-based pathway for the ultimate fixation of inorganic carbon into organic forms. With the possible exception of a low CO₂ event during the Carboniferous period 300 million years ago, estimated atmospheric CO₂ levels remained high enough to have restricted Rubisco oxygenase activity to minor levels. Only in the past 10 to 20 million years has low atmospheric CO₂ combined with abundant atmospheric oxygen to produce rates of photorespiration that would have been able to consistently inhibit photosynthesis in non-stressed plants by 35% or more above 30 °C. This would be particularly so during the Pleistocene era between 0.02 and 2 million years ago when atmospheric CO₂ fell below 200 μbar for extended periods (Figs. 2E, 2F).

By the time high rates of photorespiration became
a problem, the photosynthetic pathway centered on Rubisco was thoroughly established and intricately coordinated with metabolism throughout the plant. Thus, substantial constraints limited options for evolutionarily resolving the oxygenation problem. Oxygenation is likely an inherent feature of the reaction mechanism required for the formation of PGA from CO$_2$ and RuBP (Andrews and Lorimer, 1987), so that utilization of an entirely different carboxylation mechanism would likely be required to avoid photorespiration. While there are alternative carboxylases such as PEP carboxylase (PEPCase), their direct utilization would require a different pathway for carbon reduction and acceptor regeneration. Given this, a complete change of carboxylating enzyme and C-metabolism pathway may have been too much to accomplish in the presence of competition from already successful, albeit photosynthetically inefficient vegetation.

**B. C$_4$ Solutions to Photorespiratory Inhibition**

Instead of evolving a new carboxylase that lacked oxygenase activity, plants solved the oxygenase problem by 1) localizing Rubisco in an internal compartment that is separated from the rest of the cell or tissue by a barrier that restricts CO$_2$ efflux, and then 2) biochemically pumping CO$_2$ into that compartment using ATP. In algae, the solution to photorespiration is to localize Rubisco within an internal cellular compartment into which CO$_2$ or bicarbonate is concentrated (Badger and Spalding,
chapter 16). In terrestrial plants, photorespiration is minimized by C₄ photosynthesis, a polyphyletic system where CO₂ is first fixed by PEPCase into four-carbon (C₄) acids within photosynthetic mesophyll cells. The resulting organic acids are then transported to a bundle sheath layer where Rubisco is localized. Here, they are decarboxylated, with the result being an approximate tenfold enhancement of bundle sheath CO₂ concentration. Substantial variation is present in how various C₄ species achieve CO₂ concentration, highlighting the diverse evolutionary pathways that land plants used to arrive at a common solution to photorespiratory inhibition.

C. Variations on the C₄ Theme

All C₄ species operate on the same basic theme of pumping CO₂ via C₄ acids from the mesophyll tissue where PEPCase activity is enhanced to a bundle sheath layer where Rubisco is localized and C₄ acids are decarboxylated. Other than this, the only common feature shared by C₄ plants is a reduction in the ratio of mesophyll to bundle sheath cells when compared to C₃ plants. Because diffusion of organic acids between the mesophyll and bundle sheath must be relatively rapid, mesophyll cells in C₄ plants are rarely more than one cell distance from bundle sheath cells. As a result, mesophyll to bundle sheath cell ratios are between 1 and 2 in C₄ plants, while they are over 4 in most laminate C₃ leaves (Dengler and Nelson, 1999).

Beyond these common features, C₄ plants exhibit substantial variation in how they accomplish CO₂ concentration. In all, at least 14 distinct types of C₄ species have been identified (eight grass, three sedge, and three dicot types) and numerous more will likely be described as less common groups are studied. These variations result from three distinct decarboxylation modes and multiple patterns of anatomical modification. Biochemically, the major distinguishing feature of C₄ subtypes is the enzyme used for the decarboxylation step in the bundle sheath (Hatch, 1987). The three decarboxylation modes are NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and PEP carboxykinase (PCK). Table 1 lists economically significant C₄ plants of each subtype. Notably, economically significant C₄ plants tend to be NADP-ME species (Brown, 1999). While most C₄ species primarily use one decarboxylating enzyme, a second decarboxylating enzyme may be employed in a back-up role in some species. For example, PCK species usually have some NAD-ME activity as a means of shuttling NADH into the bundle sheath for ATP generation (Leegood and

Table 1. Economically important C₄ plant species with biochemical subtype. Modified from Brown (1999).

<table>
<thead>
<tr>
<th>Crops – grasses</th>
<th>Forages (all grasses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitaria exilis (fonio)</td>
<td>NADP-ME</td>
</tr>
<tr>
<td>Pennisetum glaucum (pearl millet)</td>
<td>NADP-ME</td>
</tr>
<tr>
<td>Saccharum officinarum (sugar cane)</td>
<td>NADP-ME</td>
</tr>
<tr>
<td>Sorghum bicolor (sorghum)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Setaria italica (foxtail millet)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Zea mays (maize, corn)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Eleusine coracana (finger millet)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Eragrostis tef (tef)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Panicum miliaceum (Proso millet)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Amaranthus edulis (grain amaranth)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Amaranthus tricolor (vegetable amaranth)</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Crops – dicots</td>
<td>Forages (all grasses)</td>
</tr>
<tr>
<td>Brachiaria mutica</td>
<td>PCK</td>
</tr>
<tr>
<td>Chloris gayana</td>
<td>PCK</td>
</tr>
<tr>
<td>Melinis minutiflora</td>
<td>PCK</td>
</tr>
<tr>
<td>Panicum maximum</td>
<td>PCK</td>
</tr>
<tr>
<td>Echinochloa colonum</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Echinochloa crusgalli</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Imperata cylindrica</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Sorghum halopense</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Digitaria sanguinalis</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Paspalum conjugatum</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Cynodon dactylon</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Eleusine indica</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Weeds – grasses</td>
<td>Weeds – sedges</td>
</tr>
<tr>
<td>Cyperus esculentus</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Cyperus rotundus</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Weeds – dicots</td>
<td>Weeds – sedges</td>
</tr>
<tr>
<td>Amaranthus retroflexus</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Amaranthus spinosus</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Portulaca oleracea</td>
<td>NAD-ME</td>
</tr>
<tr>
<td>Tribulus terrestris</td>
<td>NAD-ME</td>
</tr>
</tbody>
</table>

Rowan F. Sage and Robert W. Pearcy
Walker, 1999). Anatomically, two general distinctions are made depending upon the origin of the cell layer where Rubisco is localized. Many \( C_4 \) species localize Rubisco in a layer of parenchymatous bundle sheath cells that are derived from ground meristem. A significant number of grasses and sedges also utilize mestome sheath cells instead of or in addition to the parenchymatous bundle sheath (Dengler and Nelson, 1999). The mestome sheath is a cellular layer just inside the parenchymatous bundle sheath and is derived from procambium during development. Additional variation occurs in the metabolic steps associated with acid transport and PEP regeneration, the location and ultrastructure of chloroplasts, and properties of the wall separating Rubisco-containing cells from the mesophyll tissue (Hattersley and Watson, 1992). Many of these variations are associated with the biochemical decarboxylation step, and in some cases the association is causal. For example, in NADP-ME species, the movement of malate imports reducing power into the cell, leading to NADPH formation as a product of the decarboxylating step (Chapter 19, Leegood). As a consequence, bundle sheath chloroplasts in the NADP-ME species require less Photosystem II activity and evolve less \( \text{O}_2 \) in the bundle sheath. The reduced \( \text{O}_2 \) production within the bundle sheath allows for a bundle sheath wall that can be more resistive to \( \text{CO}_2 \) efflux, and correspondingly, NADP-ME grasses commonly have a suberized wall that hypothetically is more effective at restricting gas diffusion.

**D. Physiological Consequences of \( C_4 \) Biochemistry at the Leaf Level**

The physiological significance of the \( C_4 \) system of \( \text{CO}_2 \) concentration is best demonstrated by comparing the response of photosynthesis to the intercellular partial pressure of \( \text{CO}_2 \) and to light in \( C_3 \) and \( C_4 \) species of similar life form and ecological habitat.

**1. \( \text{CO}_2 \) Response**

Differences between \( C_3 \) and \( C_4 \) responses to \( \text{CO}_2 \) are generally so striking that they are useful indicators of \( C_4 \) photosynthesis (Downton and Tregunna, 1968; Downton, 1975). As demonstrated in Fig. 3, the \( \text{CO}_2 \) response of net \( \text{CO}_2 \) assimilation rate \( (A) \) in \( C_4 \) plants differs from the \( C_3 \) response in four main points (Edwards and Walker, 1983). 1) The \( \text{CO}_2 \) compensation point (the \( \text{CO}_2 \) level where gross photosynthesis equals respiration and thus net photosynthesis is zero) is near zero in \( C_4 \) plants at all temperatures, but is near 50 \( \mu \text{mol mol}^{-1} \) in \( C_3 \) species at moderate temperatures, rising to over 70 \( \mu \text{mol mol}^{-1} \) above 35 °C (Sage et al., 1990). 2) The initial slope of the \( \text{CO}_2 \) response of \( A \) is steeper in \( C_4 \) than \( C_3 \) plants; 3) The \( \text{CO}_2 \) saturation point occurs below 200 to 300
Theoretical Controls Over Higher Plant Photosynthesis

**Fig. 4.** Schematic CO₂ response curves of photosynthesis showing the theoretical limitations on net CO₂ assimilation rate for typical C₃ and C₄ plants.

μmol mol⁻¹ in C₄ species and is distinctly delineated. In contrast, the CO₂ saturation point in C₃ species commonly occurs at much higher CO₂ than ambient and is not sharply delineated, except at low temperature (≤15 °C; Sage and Sharkey, 1987). Finally, the CO₂ saturated rate of photosynthesis is typically lower in C₃ than in C₄ species of similar life-form. These responses lead to the commonly recognized view that C₄ species have superior photosynthetic performance at CO₂ levels below ambient while C₃ species have superior values at elevated CO₂ (Fig. 3B). These trends are, however, influenced by temperature (Berry and Raison, 1982).

Differences in the CO₂ responses of C₃ and C₄ species are explained by the limitation profiles of the respective photosynthetic types across a range of CO₂ (Fig. 4). In C₃ species, photosynthetic rates in the initial slope region of the CO₂ response curves at light saturation are generally dependent on availability of Rubisco active sites (Rubisco-limited A). In contrast, when light is limiting, the regeneration capacity of RuBP limits CO₂ assimilation (Fig. 4; von Caemmerer and Farquhar, 1981; Sage and Reid, 1994). In both cases, photosynthesis is sensitive to photorespiratory inhibition, which increases the CO₂ compensation point and reduces the initial slope of the CO₂ response curve (Edwards and Walker, 1983; Sage et al., 1990).

In C₄ plants, the initial slope of the CO₂ response is dependent upon PEP carboxylation rather than Rubisco carboxylation (Fig. 4B; von Caemmerer and Furbank, 1999). PEPCase utilizes bicarbonate rather than CO₂ as a substrate. Although the Kₘ of PEPCase for HCO₃⁻ is similar to the Kₘ for CO₂ of Rubisco (PEPCase Kₘ ~20 μM at 25 °C; Rubisco Kₘ ~10–20 μM), at cellular pH, the bicarbonate/CO₂ equilibrium favors a HCO₃⁻ concentration that is approximately eight times greater than the concentration of CO₂ (Edwards and Walker, 1983). Consequently, at CO₂ levels that are below the Kₘ for Rubisco and therefore highly limiting for RuBP carboxylation, enough HCO₃⁻ is available for high PEPCase activity. PEPCase also experiences no inhibitory side reactions such as oxygenase activity of Rubisco and its specific activity is about eight times greater than that of Rubisco (Sage et al., 1987). Thus, in the cellular environment, a mole of PEPCase can turn over about 20 to 30 times faster than a mol of Rubisco from C₃ plants. In practical terms, these differences mean that a large quantity of CO₂ can be pumped into the bundle sheath of C₄ plants so that Rubisco is substrate-saturated even at mesophyll CO₂ levels below the current atmospheric value. In C₃ plants at current atmospheric levels of CO₂, Rubisco functions at only 20% to 30% of its maximum activity at 30 °C (Sage et al., 1987).

Maximum photosynthetic rates in C₄ plants (that is, the CO₂-saturated rate) are potentially determined...
by either Rubisco activity, the rate of RuBP regeneration, the rate of PEP regeneration, PEPCase activity, or any combination thereof (von Caemmerer and Furbank, 1999). Examples where each is limiting have recently been described using transgenic Flaveria and Amaranthus species in which antisense constructs were used to modify enzyme levels (Dever, 1997; Furbank et al., 1997).

2. Quantum Yield

The quantum yield of photosynthesis is the ratio of photosynthetic product (µmol CO₂ taken up or O₂ released) expressed relative to absorbed light intensity. At very low light (for example, less than 100 µmol photons m⁻² s⁻¹ (< 5% of full sunlight), a maximum proportion of absorbed photons is used for photosynthetic carbon fixation and the metabolism of photorespiratory products. At these photon fluxes, the initial slope of the light response of photosynthesis is linear and reflects the maximum quantum yield of CO₂ fixation. At higher light intensities, light absorption exceeds light utilization by the cellular photochemistry. When this occurs, the excess light energy is dissipated as heat causing the instantaneous quantum yield to decline because absorbed light

Fig. 5. A. Variation in quantum yield of C₃ (left side) and C₄ plants (right side) among photosynthetic subtypes and between monocots and dicots. The top and bottom of the boxes give the 75th and 25th percentile, respectively, and the line through the center gives the median value for each. Error bars express the 10th and 90th percentile. The open and shaded boxes are for dicots and monocots, respectively. B. The dependence of quantum yield on temperature. The line gives the typical temperature dependence of quantum yields in C₃ species. Quantum yields are independent of temperature in C₄ species; the diagonally hatched areas give the range of values in quantum yield shown in panel A. The intersection of the line for C₃ species and the ranges for the C₄ species shows the range of crossover temperatures possible. (Redrawn from data in Ehleringer and Pearcy, 1983).
energy is not used in the biochemical reactions of photosynthesis (Björkman and Demmig-Adams, 1994; Genty and Harbinson, 1996).

In studies of C₄ photosynthesis, the maximum quantum yield has become an important index of photosynthetic performance because it directly reflects the energy costs of the two photosynthetic pathways. The major energetic cost of the C₄ pathway is the two extra ATP per CO₂ fixed that are required to regenerate PEP. In the absence of photorespiration, these energy costs reduce the maximum quantum yield of C₄ plants by 30% to 40% relative to C₃ plants (Fig. 5A). Under current atmospheric conditions of 360 μmol mol⁻¹ CO₂, 210 mmol mol⁻¹ (21%) O₂, and 20 to 30 °C, energy losses due to photorespiration in C₃ leaves are approximately equivalent to the additional energy costs of the C₄ concentrating mechanism. Thus quantum yields are similar (Fig. 5A). Because of the temperature, CO₂ and O₂ dependence of photorespiration, the quantum yields of C₄ plants also exhibit a high dependence on these factors. At current atmospheric CO₂ contents, maximum quantum yields tend to be greater in C₄ than C₃ species above 30 °C, but lower below 25 °C (Fig 5B). Reducing atmospheric CO₂ shifts the equivalency point to lower temperature, such that at CO₂ levels encountered during the Pleistocene (~200 μmol mol⁻¹), the temperature where quantum yields of C₃ and C₄ species are equal would be less than 20 °C (Ehleringer et al., 1997). In contrast to C₃ plants, for a given C₄ species, maximum quantum yields are constant, showing no temperature, CO₂ or O₂ dependency (Fig 5B). There is, however, significant variation in quantum yields between the various classes of C₄ plants.

### 3. Variation of Quantum Yield of C₄ Species

When expressed on the basis of absorbed quanta, the quantum yields of C₃ species under non-photorespiratory conditions have been found to be remarkably invariant (Björkman and Demmig, 1987). By contrast, in C₄ species, considerable difference exists between species of varying decarboxylation type and evolutionary origin. NADP-ME plants have higher quantum yields than NAD-ME plants of similar taxonomic group, and within the NADP-ME and NAD-ME decarboxylation types, grasses consistently have somewhat higher quantum yields than dicots (Fig 5A; Ehleringer and Pearcy, 1983).

The reasons for differences in quantum yield between decarboxylation types have proved elusive to identify. The costs for CO₂ concentration in NADP-ME and NAD-ME type plants consist of 2 ATP per CO₂ fixed for regeneration of PEP, plus the amount of ATP required to pump in extra CO₂ (overcycle CO₂) to compensate for the CO₂ that leaks out of the bundle sheath. In PCK type plants, one extra ATP and 0.5 extra NADPH are required in addition to the amount required for overcycling (Hatch, 1987).

In the absence of overcycling, there is little theoretical difference among C₄ decarboxylation types in the energy required for the CO₂ concentration cycle. However, leakage is unavoidable because the numerous plastomes required for C₄ transport provide a low resistance path for diffusive efflux relative to the surrounding wall region of the bundle sheath (Hatch, 1987). The degree of leakage may also vary between photosynthetic subtypes because of the need to allow for efflux of any O₂ produced by Photosystem II (PS II) activity in the bundle sheath (BS) cells. NADP-ME species have little PS II activity in the BS chloroplasts, and therefore have little need to allow for O₂ efflux. In contrast, NAD-ME and PCK species have substantial PS II activity in the BS tissues and thus it was initially hypothesized that plants of these subtypes have bundle sheath walls that were more permeable to gaseous efflux than NADP-ME species (Farquhar, 1983). However, estimates of leak rates show little correlation between C₄ subtype and quantum yield. The fraction of CO₂ produced in the bundle sheath that actually escapes is difficult to quantify and estimates have ranged from 0.08 to 0.5 (Farquhar, 1983; Henderson et al., 1992; Hatch et al., 1995). The upper value, based on measurement of carbon isotope discrimination in leaf tissue, would be energetically costly because it would require two turnovers of the C₄ cycle, and hence two extra ATP, for each CO₂ assimilated. It may, however, be an overestimate because of other, non-photosynthetic sources of isotopic discrimination in leaf tissues (Henderson et al., 1992). Short-term fractionation studies, which should reflect photosynthetic processes, yielded leakage estimates close to 0.21 for a wide range of species of different C₄ types (Henderson et al., 1992). Recently, application of a new pulse-chase technique for direct measurement of the leak rate gave values for 11 species ranging from 0.08 to 0.14 (Hatch et al., 1995). At the present time, the consensus is that the leak rate is generally between 10% to 20% of the net CO₂ assimilation rate but it is not dependent upon bundle-sheath wall properties such as the degree of suberization, nor on
C₄ subtype. Thus, permeability differences do not explain variation in quantum yields between C₄ subtypes.

An alternative possibility is that variation in quantum yields is related to differences in the efficiency of light absorption and partitioning of quanta between bundle sheath and mesophyll cells. Differences in quantum yields between monocots and dicots have been attributed to a less optimum spacing between vascular bundles and therefore to a lower efficiency of light absorption (Ehleringer et al., 1997). This is an intriguing possibility that requires experimental verification.

III. Primary Environmental Controls—Temperature and Light

The geographic distributions of C₄ plants is highly dependent upon temperature during the growing season, and light regimes, so much so that these parameters can be considered primary controls over the distribution of C₄ plants. They effectively override influences of other environmental parameters such as water or soil nutrient supply. C₄ plants require warm to hot growing seasons and with only a few exceptions, do not occur in deeply shaded environments. While the mechanistic causes of these distribution patterns arise from the differential responses of C₃ and C₄ photosynthesis to light and temperature, numerous ecological factors, notably disturbances such as fire, interact with physiological differences to control where, and when, C₄ plants are successful. In this section, we discuss light and temperature controls over C₄ plant distribution, describing first the pattern of C₄ distribution along these environmental gradients, followed by a discussion on how photosynthetic properties may explain the trends.

A. Temperature

1. Geographic Distribution

a. Grasses

The first distinctive environmental characteristic noted for C₄ photosynthesis was its high representation among grasses of tropical origin and summer crops, weeds, and forages (Downton and Tregunna, 1968; Black et al., 1969). Subsequent work has shown that C₄ grass abundance is highly correlated with temperature along latitude, altitude and seasonal gradients. Below approximately 25° latitude, both grass floras and grassland primary productivity are dominated (>75% representation) by C₄ species. Only where woody species are established will C₄ productivity be minimal or absent. With the exception of species adapted to salinized, sandy, or arid soils, tree and shrub species are almost exclusively C₃.

In the low elevation tropics, C₃ grasses are uncommon in open grasslands and deserts but increase in importance in tropical marshes and swamps. They dominate grass floras under forest canopies and at high elevation (Skerman and Riveros, 1990; Sage et al., 1999b). The shift from C₄ dominated to C₃ dominated grasslands along altitude gradients always occurs above 1000 m, but the precise altitude of crossover varies with precipitation. In wet tropical areas such as Hawaii and New Guinea, C₄ grasses dominate the grass cover up to 1500 to 2000 m (Rundel, 1980; Bird et al., 1994). On Mt. Kenya in East Africa, by contrast, C₄ grasses can predominate up to 2700 m, and can be locally dominant at even higher elevations on the drier microsites (Tieszen et al., 1979; Young and Young, 1983). However, above 3000 to 3500 m, C₃ cover and floristic representation always predominates over that of C₄ plants (Sage et al., 1999b).

Altitude limits of C₄ grasses in natural vegetation are modified by competitive interactions with both C₃ grasses and woody species. In agricultural systems, human management offsets much of the competitive effects from C₃ vegetation and the physiological potential for production becomes a major control over crop distribution. In montane regions at low latitudes, C₄ crops predominate at low elevation, but give way to C₃ crops and forages at high elevation (Skerman and Riveros, 1990). In the Ethiopian highlands, for example, C₄ crops predominate below 1500 m, but are rarely cultivated above about 2500 m (Fig. 6). Instead, many temperate-zone crops and forages are grown in higher elevations in the tropics. In East Africa, C₃ ryegrass (Lolium perenne) and Festuca spp become important forage above 2200 m. Below this elevation, all the major forages are C₄ even on irrigated soils (Booman, 1993).

At latitudes above 55°N and 46°S, C₄ grasses are rare at all elevations. At 50°N, C₄ grasses make up between 5% to 15% of regional grass floras, but are rarely dominant except in localized situations where microsites exhibit a combination of factors favorable to C₄ vegetation (Sage et al., 1999b). Microsites with
high solar insolation, saline and/or dry soils, and slope aspects facing the equator favor plants in generally cool climates (Guy et al., 1986; Schwarz and Redman, 1988; Pyankov and Makronosov, 1993). In many regions, species of high latitude are largely weedy species dependent upon human action for their continued success. When weeds are factored out, the contribution to a regional grass flora declines by 50% to 100%. In the British Isles, for example, 90% of the grass flora is introduced (Stace, 1997).

Between 50°N and 30°N, and 45°S and 35°S, the contribution of \( C_4 \) grasses to grassland cover and productivity increases with decreasing latitude until grasses predominate at the warmer ends of the temperate zones (Pyankov and Mokronosov, 1993; Bird and Pousai, 1997; Tieszen et al., 1997). In this zone, the latitude at which \( C_4 \) grasses become dominant is variable, and reflect interactions with other environmental factors (Epstein et al., 1997).

### b. Sedges and Dicots

\( C_4 \) sedges generally do not show the same degree of ecosystem dominance as do \( C_4 \) grasses. This is probably because sedges (plants of the Cyperaceae) are more likely to occur on flooded soils that also support a rich \( C_3 \) sedge flora, even at low latitudes. While the proportional representation of \( C_4 \) sedges shows similar latitudinal responses as \( C_4 \) grasses, they do not extend to as high of latitude or elevation and they have reduced representation within a sedge flora than do \( C_4 \) grasses within the grass flora from the same region (Sage et al., 1999b). For example, in localities where \( C_4 \) grasses may represent over 80% of the grass flora, \( C_4 \) sedges may represent only 40% to 60% of the sedge flora (Teeri and Stowe, 1976; Teeri et al., 1980).

\( C_4 \) dicot distribution is more difficult to characterize in terms of environmental correlates than grasses and sedges because \( C_4 \) dicots rarely account for more than 5% of the dicot flora. Thus variation in \( C_4 \) dicot estimates is often due more to variation in total dicot species, rather than \( C_4 \) dicot numbers. Instead, presenting the occurrence of \( C_4 \) dicots in their respective families or functional groups is generally more meaningful. When this is done, similar latitudinal patterns as exhibited by grasses become apparent. In the Chenopodiaceae, less than 20% of the species in Europe north of the Mediterranean basin are \( C_4 \). In the Mediterranean basin, approximately 50% of the Chenopods are \( C_4 \) while over 95% are \( C_4 \) in central Africa (Akhani et al., 1997). Similarly, along the Nile river in Egypt, the proportion of species in the Euphorbiaceae that are \( C_4 \) rises from less than 20% along the Mediterranean sea in Northern Egypt, to 50% in southern Egypt near the Sudanese border (Batanouey et al., 1991).

### 2. Seasonal Trends

In temperate climates where \( C_4 \) species are abundant, their activity is nearly always centered on the summer months. Typically, they break dormancy some three to six weeks after \( C_3 \) species of similar functional groups. Seeds of annual \( C_3 \) weeds germinate in early to mid-spring, while \( C_4 \) weeds germinate mid-to-late spring (Baskin and Baskin, 1977). Similarly, in the Sonoran desert, \( C_3 \) species germinate under the temperatures and photoperiods characteristic of autumn and winterwhile \( C_4 \) annuals germinate under late-spring conditions (Mulroy and Rundel, 1977; Kemp, 1983). Among grasses from the Great Plains of North America, \( C_3 \) grasses break dormancy in March to April, while \( C_4 \) grasses appear late April to May (Dickinson and Dodd, 1976; Ode et al., 1980; Monson et al., 1983; Sage et al., 1999b). The result of these differences is the long-recognized segregation...
of grasses and weeds into warm season (largely C₄) and cool season (largely C₃) species, and management practices have adjusted to account for these physiological differences (Skerman and Riveros, 1990; Boonman, 1993)

3. Temperature Thresholds

It is now well recognized that growth season temperature is the principle controlling factor over the abundance of C₄ plants along latitude and elevation gradients, particularly with grasses and sedges. In North America and Australia, C₄ grass and sedge distribution is well correlated (r² > 0.8) with numerous indices of growth season temperature including mean temperature of the warmest month, mean minimum temperature of the warmest month, mean maximum of the warmest month, number of days above 32 °C, summer pan evaporation, and potential evapotranspiration (Teeri and Stowe, 1976; Hattersley, 1983; Epstein et al., 1997). Close correlation between C₄ abundance and growth season temperature has also been observed along altitude gradients (Long, 1983; Cavagnaro, 1988). In the tropics, annual temperature is a good index of C₄ abundance along altitude gradients while in temperate zones annual temperature is less meaningful because winter temperature is poorly correlated with summer temperature. Winter cold does not appear to be any more harmful to dormant C₄ species than their dormant C₃ associates (Long, 1983; Schwarz and Reaney, 1989).

From analysis of geographical gradients, the following temperature thresholds are critical for C₄ plants. Below mean minimum temperatures of the warmest month of 6 to 10 °C, C₄ plants are largely absent (although a few hardy species can survive nightly lows averaging 2 °C) (Hattersley, 1983; Long, 1983; Pyankov and Mokronosov, 1983; Sage et al., 1999b). Transition from C₃ to C₄ dominance in grass floras occurs at minimum temperatures of the warmest month of 15 °C to 18 °C; and mean daily temperatures of about 22 °C (Teeri and Stowe, 1976; Hattersley, 1983; Bird and Pousai, 1997). Above mean daily temperatures of 24 °C, C₄ plants largely dominate a regional grass flora of non-shaded habitats.

4. Physiological Explanations for the Temperature Dependency

Rarely in ecology do single physiological mechanisms explain distinct biogeographical patterns. In the case of the correlation between C₄ dominance and temperature, a strong argument can be made that the underlying mechanism explaining the trend is a result of the distinct temperature response of photosynthesis in C₄ plants relative to C₃ species of similar life form.

a. The Temperature Response of C₄ Relative to C₃ Photosynthesis

The temperature response of net CO₂ assimilation in C₄ and C₃ species show distinct differences: C₄ species have a pronounced rise in assimilation between 10 °C and the thermal optimum for photosynthesis, which usually occurs between 30 °C and 40 °C (Fig. 7; Björkman et al., 1975, 1980). Atmospheric CO₂ variation has little effect on this response above the current ambient of 365 μmol mol⁻¹, but reductions in CO₂ below 365 μmol mol⁻¹ reduce the rate of C₄ photosynthesis at the thermal optimum. In C₃ species, the temperature response of photosynthesis is highly dependent upon CO₂ partial pressure at all temperatures. At low CO₂ partial pressures, the temperature response of photosynthesis is relatively flat, which reflects a high control over photosynthetic rate by Rubisco. Rubisco has low thermal responsiveness at CO₂ partial pressures below its Kₐ, because both the Kₐ and Vₘₐₓ have similar thermal dependencies (Berry and Raison, 1981). At elevated CO₂, by contrast, C₄ photosynthesis shows a high thermal dependency at temperatures below the thermal optimum, similar to C₃ species. This is because the RuBP regeneration capacity, which has a high thermal dependency (Q₁₀ near 2) dominates the control of CO₂ assimilation in C₃ species at elevated CO₂ (von Caemmerer and Farquhar, 1981; Berry and Raison, 1981).

Comparison of the thermal responses of C₄ and C₃ species show that at low temperature (<18 °C), C₃ species typically have equivalent or higher CO₂ assimilation rates than ecologically similar C₄ species, even at the low CO₂ levels of the recent geological past. At elevated temperature, the C₄ species outperform C₃ species, especially at the low CO₂ level of the past 20,000 years (200 to 270 μmol mol⁻¹). The superiority of C₄ photosynthesis at elevated temperatures is commonly accepted as an important mechanism promoting C₄ success. Growth and competition studies commonly show that where temperatures favor C₄ photosynthesis, the overall performance of C₄ species is superior to C₃ species of similar life form (Table 2; Peary et al., 1981; Christie...
and Detling, 1982; Grise, 1996). In turn, superior \( C_3 \) photosynthesis at cooler temperature is well correlated with superior \( C_4 \) performance at the whole plant level (Table 2).

**b. Why Do \( C_4 \) Plants Fail in Cool Climates?**

The reasons for failure of all but a few \( C_4 \) species to grow in climates where growing season minimums average below ~8 °C has not been resolved and continues to be discussed at length (for example, Long, 1999). Most \( C_4 \) species are chilling sensitive, leading to speculation that inherent properties in the \( C_4 \) syndrome may predispose \( C_4 \) plants to chilling injury (Long, 1983). For example, PPDK and PEPCase can dissociate upon exposure to low temperature, indicating the \( C_4 \) pump may be sensitive to chilling (Leegood and Edwards, 1996). Alternatively, chilling intolerance may simply reflect the tropical origin of most \( C_4 \) species, especially the economically important ones such as maize that have been heavily studied. If true, \( C_4 \) plants from high altitude and latitude may not exhibit chilling sensitivity. Research on \( C_4 \) plants from the low temperature extremes of the \( C_4 \) distribution support the latter possibility, as little evidence exists that they are damaged by low temperature any more than their \( C_3 \) neighbors. Enzymes of the \( C_4 \) pump from high latitude and altitude plants are relatively cold stable in low temperature, and the degree of photoinhibition is less in chilling conditions when compared with low latitude relatives (Simon and Hatch, 1994; Leegood and Edwards, 1996; Matsuba et al., 1997; Pittermann, 1998; Long, 1999).

A common viewpoint is that \( C_4 \) plants perform poorly in cool climates; however, this may not reflect physiological possibilities, because if given adequate resources, high altitude \( C_4 \) species perform very well, and can even exhibit productivity in excess of local \( C_3 \) crops. Beale and Long (1997) and Kao (1997) for example, report that *Miscanthus* species grown in cool temperatures in Europe and Asia can exhibit \( CO_2 \) assimilation rates and yields exceeding those of many \( C_3 \) species of similar functional type. Similarly, one of us (RFS) observed in the alpine zone of White Mountains, California, that robust swards of the montane \( C_4 \) grass *Muhlenbergia richardsonis* occurred within 200 m of its known altitude limit in North America of 3950 m.

Given these observations, the failure of \( C_4 \) species to grow in cold environments with growth minima below about 8 °C appears to reflect competitive suppression caused by \( C_3 \) species (as seen by Pearcy et al., 1981, and Christie and Detling, 1982). The dominance of the \( C_3 \) species is probably due in large part to their higher photosynthetic capacity observed in low temperature (Fig 7), and their superior quantum yields. This likely gives the \( C_3 \) plants more of an edge in the competition for other scarce resources, and thus prevent \( C_4 \) species from acquiring the resources they need to exhibit the strong performance that can be observed in cool climates when they are grown in isolation under resource rich conditions.
In warm environments, the major C₃ functional groups that are successful at the expense of C₄ plants are the shrubs and trees of forests and scrubland. While much of the competition between grasses and woody species is affected by disturbance factors such as fire and browsing, modification of the light environment by the woody canopy is a primary determinant of their ability to suppress vegetation. In plants, access to moderate to high light is, along with warm temperature, a prerequisite for the dominance of C₄ species.

1. Distribution Along Light Gradients

‘Moderate to high light’ is vague, but necessary, terminology because it is difficult to precisely state the minimum light requirement for C₄ success. Full sunlight to modest shade (that is, midday photon flux densities greater than about 1000 μmol photons m⁻² s⁻¹) fulfill the light requirement for C₄ success wherever C₄ plants are found. C₄ vegetation shows little reduction in species coverage or biomass contribution until shade intensities below 50% of full sunlight (Sage et al., 1999b). In the shade of trees and shrubs in tropical savannas, C₄ grasses remain abundant down to at least 30% of full sunlight values, and often show an increase in abundance in partial shade between 40% and 60% of full sun (Belsky et al., 1989; Weltzin and Coughenour, 1990). In a northern mixed grass prairie/savanna of Minnesota and Wisconsin, C₄ grasses begin to have less coverage relative to open sites only below 50% of canopy openness and they become rare below about 25% of canopy openness. (Bray, 1958; Ko and Reich, 1993; Means, 1997). C₄ plants are almost always absent in full shade of a forest canopy, where daily light intensities are typically 10% or less than the light availability in open areas (Medina and Klinge, 1983; Pearcy, 1990).

Shade adaptation has been reported for a number of C₄ grasses. For example, the genera Microstegium, Muhlenbergia, and Paspalum each have numerous species that are found in deep shade (Brown, 1977; Smith and Wu, 1994; Horton and Neufeld, 1998). However, examination of the microsite descriptions of these shade-adapted species indicates that they often grow on sandy soils, or drought prone sites (Brown, 1977). Such areas often have slightly more open overstories where light intensity may be higher than 10% of full sky exposure. Several species of Chaemasyce (=Euphorbia) are native to the understory of moist Hawaiian forests. Some of these species (for example, Chaemasyce forbesii) have shade adaptation syndromes equivalent to co-occurring C₃ understory species (Pearcy and Calkin, 1983). The occurrence of shade adaptation in Chaemasyce species may reflect early invasion of the Hawaiian Islands by high-light-adapted ancestors of the current shade-adapted C₄ species. Because of the remoteness of the Hawaiian archipelago, shade-adapted C₃ species may have been rare early in the islands formation, thereby leaving open understory niches for colonization by C₄ Chaemasyce species. In the absence of competition from C₃ shade specialists, the C₄ invaders are hypothesized to have eventually evolved shade adaptations characteristic of most understory specialists (Robichaux and Pearcy, 1980; Pearcy and Calkin, 1983).

Little has been reported on the light requirement for ecological success of C₄ sedges and dicots. C₄...
sedges probably show similar responses as grasses, given that they are often found among dense vegetation in tropical wetlands. C₄ dicots appear to have higher light requirements than grasses and sedges based on quantum yield comparisons (Ehleringer and Pearcy, 1983). With the exception of Chaemasyce spp, C₄ dicots are rare in understory environments.

The occurrence of shade-adapted C₄ species demonstrates that the C₄ syndrome does not preclude the growth and survival of C₄ plants in low light environments; however, the rarity of C₄ plants in forest shade indicates they are poor competitors against C₃ plants in low light environments. This poor competition is not simply a matter of grasses against woody functional types, because C₄ dicots are often woody, and some develop into robust shrubs and small trees. Moreover, hundreds of C₃ grass species are shade-adapted understory species, demonstrating that the grass functional type is able to adapt well to forest shade (Renvoize and Clayton, 1992).

What then, might explain the general absence of C₄ species in forest shade?

2. Physiological Controls

a. Photosynthetic Capacity

Early comparisons of C₃ and C₄ species commonly showed C₄ plants had substantially higher light saturated rates of photosynthesis and higher light saturation points (see Pearcy and Ehleringer, 1984 for review). However, much of the early work was based on comparisons between species of different growth form or habitat requirements; for example, temperate versus tropical crop species. When ecologically similar C₄ and C₃ plants are compared at moderate temperatures (25 to 30 °C) and current ambient CO₂ pressures, there is often little difference in light-saturated photosynthetic rates and light saturation points (Pearcy and Ehleringer, 1984). Desert annuals, weeds and annual crop species are known to have the highest photosynthetic capacities, and within each of these groups C₄ and C₃ species exhibit substantial overlap, with photosynthesis of either group often exhibiting light responsiveness at full sunlight (Pearcy and Ehleringer, 1984; Sage and Pearcy, 1987). In turn, many C₄ plants perform well in shaded environments, where light intensities may not commonly exceed 30% of full sun. Consequently, at moderate temperature, there is little obvious difference in photosynthetic light requirements at the leaf level. At higher temperature (30 to 40 °C), however, increasing rates of photorespiration limit photosynthetic rates in C₃ species, while photosynthesis in C₄ species is stimulated. Under these conditions, C₄ species will typically exhibit higher light saturation points than C₃ species and superior photosynthetic performance at subsaturating light intensities. In turn, at cooler temperatures (<20 °C), C₃ species often have higher light requirements for photosynthetic saturation.

These differences in light response largely reflect the ability to exploit an abundance of light energy by C₄ plants. In forests and shrub understories, by contrast, carbon balance is most dependent upon low light performance, which largely reflects quantum yield, respiration, and the ability to exploit brief, high light events (sunflecks).

b. Quantum Yield and C₄ Performance in Shade

The rarity of C₄ photosynthesis in the shade has been attributed to a disadvantage in C₄ quantum yield relative to those of C₃ species (Ehleringer, 1978). In deep shade, quantum yields are important determinants of photosynthetic rate, and species with higher quantum yields should have higher photosynthetic rates in species, while photo synthesis in C₄ species is stimulated. Under these parameter alone, the failure of C₄ species to occupy forest understories can be partially understood. Forest understories rarely experience thermal regimes that are highly favorable to C₄ species because understory temperatures do not commonly rise above 30 °C, even in the tropics (Pearcy and Calkin, 1983). Forest understories also experience elevated atmospheric CO₂ levels as a result of decomposition of forest detritus, which can increase the average daytime CO₂ level by 10% and with it the advantage of the C₃ quantum yield (Bazzaz and Williams, 1991). Understories are also more humid, favoring stomatal opening and relatively high intercellular CO₂ concentrations. Together, these factors contribute to potentially superior C₃ quantum yields and photosynthetic performance in the typical deep shade of the understory environment.

c. Respiratory Costs

Net carbon acquisition in low light is also influenced by respiratory costs for building and maintaining photosynthetic tissues. Species with high leaf respiration rates do poorly in shade, because it
The Physiological Ecology of $\text{C}_4$ Photosynthesis

subtracts from the carbon available for the remainder of the plant. It has been hypothesized that because $\text{C}_4$ photosynthesis has lower protein requirements, it may have reduced respiratory costs (Byrd et al., 1992). Byrd et al. evaluated this in a number of high light adapted $\text{C}_3$ and $\text{C}_4$ species, and found no evidence of respiration differences based on photosynthetic capacity. Respiratory costs have not been compared in shade adapted $\text{C}_3$ and $\text{C}_4$ species, although available evidence from light response curves indicate differences in dark CO$_2$ exchange and light compensation points are not striking (Pearcy and Calkin, 1983; Horton and Neufeld, 1998).

d. Exploitation of Sunflecks

Variation in irradiance because of cloud movement and sunflecks in leaf canopies are important controls over daily carbon gain in most field environments. In shade-adapted $\text{C}_3$ species, 30% to 70% of daily carbon gain is typically acquired from sunflecks (Pearcy, 1990). The ability to exploit sunflecks is an important factor determining performance in low light environments, with sun-adapted species having a reduced ability to utilize sunflecks relative to shade-adapted species (Sharkey et al., 1986). Could this explain the general absence of $\text{C}_4$ species from deep shade?

The basic responses to transient light differ between $\text{C}_4$ and $\text{C}_3$ species because of the absence of a photorespiratory CO$_2$ burst in $\text{C}_4$ species and, as demonstrated for maize, a burst of CO$_2$ evolution at the beginning of short (1–10 s) sunflecks (Krall and Pearcy, 1993). The photorespiratory burst has no net effect on sunfleck utilization in $\text{C}_3$ species but the CO$_2$ burst at the beginning of short sunflecks in maize substantially reduces the efficiency with which sunflecks are utilized by this species. The burst may be due to imbalances between metabolite pools and reducing power created during short sunflecks in the bundle sheath of NADP-ME species, such that some of CO$_2$ initially fixed into $\text{C}_4$ acids in the mesophyll cannot be refixed within the bundle sheath. For longer sunflecks, no initial burst is present, presumably because metabolite pools and energy supplies have had sufficient time to come into balance. Although there is a substantial restriction in the ability to utilize short sunflecks in maize, the overall consequence may not be that great. Short sunflecks, although present in significant numbers, contribute much less of the total PFD than longer sunflecks, and therefore inefficiencies in their use would not be a significant limitation to carbon gain in understory microenvironments. Indeed, its significance may be somewhat greater in crop canopies where a larger fraction of the available PFD is contributed by short sunflecks. Utilization of longer sunflecks is qualitatively similar in $\text{C}_4$ and $\text{C}_3$ species (Pearcy et al., 1985); with most of the quantitative differences being related to the faster stomatal responses in $\text{C}_4$ grasses as compared to $\text{C}_3$ dicots (Fay and Knapp, 1995), or to differences in photosynthetic capacity.

Comparisons of shade-adapted understory species, such as *Chamaesyce forbesii* ($\text{C}_4$) and an ecologically similar $\text{C}_3$ tree, *Claoxylon sandwicense* led to the conclusion that $\text{C}_4$ photosynthesis was adaptively neutral relative to $\text{C}_3$ photosynthesis in the Hawaiian forest understory where they occur (Pearcy and Calkin, 1983). No advantage in utilization of sunflecks for photosynthesis or growth could be associated with the $\text{C}_4$ pathway in *Chamaesyce forbesii*. More recently Horton and Neufeld (1998) examined shade performance of *Microstegium vimineum*, a shade-tolerant $\text{C}_4$ grass that has become a noxious weed in warm-temperate zones of eastern North America. This species does well in moist, low-light habitats generally favorable to $\text{C}_3$ plants, where its aggressive expansion is somewhat of a surprise. *Microstegium vimineum* exhibits typical shade-adaptations often found in $\text{C}_3$ plants adapted to low light conditions but some important differences indicate it may be more of a gap species than true deep shade species. First, the induction state of photosynthesis declined far more rapidly in *M. vimineum* than is apparent in $\text{C}_3$ species from understory environments. This could lead to substantially less photosynthetic carbon gain in environments where sunflecks are separated by 2 to 5 min, as is often the case. Second, stomatal conductance tended to track light variation as is observed in gap-adapted species. While this saves water, it could lead to reduced exploitation of light flecks in rapidly changing light environments. In contrast to *M. vimineum*, understory $\text{C}_3$ species maintain open stomates between sunflecks, thereby avoiding transient stomatal limitations (Horton and Neufeld, 1998).

3. Synopsis

As with temperature, differences in quantum yields, respiration and sunfleck utilization do not indicate an obvious reason why $\text{C}_4$ species are ecologically
unfit in low light habitats. While C₄ plants are able to exploit low light, the temperature and CO₂ conditions generally do not favor greater low light performance of C₄ plants, but will often favor slightly higher C₃ quantum yields. Second, C₄ species can exploit dynamic light environments effectively, but may have a slightly reduced ability to exploit short sunflecks, and because of more rapid loss of induction state, may not be as effective exploiting intermediate sunflecks. Taken together, these slight reductions in C₄ performance relative to C₃ may be enough to shift the competitive balance in favor of C₃ species except where other factors such as poor soil fertility or drought may offset any C₄ advantage.

The exclusion of C₃ species from forests has great ecological significance, because where woody C₃ species can become established, they will, with sufficient time, become dominant. To some degree, the ability of woody species to establish and persist in grasslands uncouples ecological success from photosynthetic pathway. Establishment is not solely a consequence of photosynthetic potential, but is often more a function of stress tolerance and life history characteristics. In turn, persistence depends on the disturbance regime of the landscape. Ecological disturbances that select against woody plants, namely fire, severe drought, large animal herbivory, wind, floods, and human activity are critical modifiers of woodland distribution and are often a prerequisite for C₄ success (Sage et al., 1999b). This understanding has often led ecologists to ignore photosynthetic pathway as an important control over grass/woodland dynamics. It is important, however, to realize that ecological factors that affect woodland success often act by modifying light competition between interacting C₃ and C₄ vegetation.

IV. Secondary Environmental Controls—Water Supply, Salinity, Nitrogen

In addition to growth season warmth and moderate to high light requirements, the success of C₄ species is influenced by water availability, salinity and soil nitrogen (N) supply. Here, we discuss the physiological basis for the different responses of C₃ and C₄ species to water supply, salinity, and drought, and then examine how these differences modify the geographical patterns established by temperature and light.

A. Water Availability

1. Physiological Considerations

Differences in performance along gradients of water availability can arise if species have inherently different tolerances of extremes of water supply, or have different efficiencies of water use. C₃ and C₄ plants have no inherent differences in their tolerance of drought or flooding (Pearcy and Ehleringer, 1984). In response to drought, they show similar levels of physiological impairment at similar water potentials or relative water contents as C₃ species of similar life form. There are, however, consistent differences between C₃ and C₄ species in the efficiency with which water is used that result from the ability of the C₄ cycle to concentrate CO₂. These differences promote C₄ over C₃ vegetation in specific situations.

Instantaneous water use efficiency is expressed as the ratio of photosynthesis to transpiration. Assuming all things are equal, the higher water use efficiency in C₄ plants occurs because the relative stomatal aperture (indexed by stomatal conductance) is regulated at a lower value in C₄ than C₃ species having the same CO₂ assimilation capacity. This is possible because the CO₂ concentration system allows C₄ plants to pump enough CO₂ into the bundle sheath to saturate Rubisco, even at intercellular CO₂ levels in the mesophyll tissue as low as 100 μmol mol⁻¹. On average, C₄ plants will have stomatal conductance values that are 50% to 70% less than C₃ species with identical CO₂ assimilation rates (Schulze and Hall, 1982; Sage and Pearcy, 1987). Assuming equivalent leaf temperatures, this will translate into transpiration rates that are 50% to 70% less in the C₄ than C₃ and WUE values that are two to three times higher. However, because of the lower level of evaporative cooling, C₄ leaf temperatures are on average higher, increasing transpiration and reducing the WUE differences between C₄ and C₃ plants in field situations.

High water savings can also be achieved through stomatal closure in C₃ plants but this comes at substantial reductions in photosynthetic capacity. Except at low temperature (<15 °C), C₃ photosynthesis decreases proportionally with reduction in stomatal conductance, reflecting the reduction in CO₂ supply to the chloroplast (Björkman, 1976; Farquhar and Sharkey, 1982). C₃ species that attempt to save water through stomatal closure lose much of their potential for carbon gain and experience high
levels of photorespiration, which reduces nutrient and light use efficiency. Thus, in environments where water is limiting, C₃ plants that are conservative in their water use may compete with C₄ plants that have similar water demands, but twice the CO₂ acquisition potential and therefore a much greater growth capacity. This greater growth potential may enable the C₄ to produce roots and leaves faster, thereby capturing the remaining water, nutrient, and light resources in the environment.

Differences in WUE between C₃ and C₄ species are most significant in warmer climates because the transpiration demand rises exponentially with increasing temperature. In warm-climate C₃ plants, stomatal closure for water conservation combines with thermal enhancement of photorespiration to give a synergistic reduction in CO₂ assimilation capacity. In C₄ plants, rising temperatures stimulate PEPCase and the other enzymes of the C₄ pump so that the ability to supply Rubisco with high levels of CO₂ is increased, even at the lower intercellular CO₂ levels that may result if stomatal aperture was reduced. As a consequence, rising temperature can stimulate photosynthesis in C₄ plants at low intercellular CO₂, while in C₃ species, increasing temperature above 30 °C reduces the rate of net CO₂ assimilation at low intercellular CO₂ levels (Fig. 8; Björkman, 1976; Björkman et al., 1980). Correspondingly, WUE in C₄ species can rise with increasing temperature above 30 °C, while in C₃ plants it will almost always decline.

2. Geographical Distribution

Within a few years of the discovery of the C₄ syndrome, the large water use efficiency differences between C₃ and C₄ plants were noted, as was a pattern where C₄ species often predominated in hot, dry environments (Black, 1973). The associations between an apparent drought adaptation (higher WUE) and occurrence in arid sites was instrumental in the development of the textbook paradigm that photosynthesis is an adaptation for aridity (for example, see Raven et al., 1992). This view has some merit, in that greater water use efficiency can improve C₄ performance relative to C₃ in arid environments, possibly enabling C₄ species to occur in climates where temperature and light might otherwise favor C₃ species. For example, at the cold end of their distribution range, C₄ grasses are commonly noted to occur on dry sites, or sandy soils with reduced waterholding capacity. In central Asia and in Canada, for example, the native C₄ species occurring north of 50° largely occur in arid sites (or saline soils, which will be discussed below) (Schwarz and Redman, 1988; Pyankov and Mokronosov, 1993). C₄ species occurring above 3500 m in Asia, North America and the Peruvian Andes are also reported to occur on xeric soils (Sage et al., 1999b). On Mount Kenya, Young and Young (1983) show that in the zone of approximate equivalent frequency, C₄ grasses occurred on significantly drier sites than C₃. In each of these instances, however, the direct role of aridity versus other microclimate factors has to be resolved. For example, increasing aridity brings about a gradual loss of canopy cover, thus opening the canopy and enabling more light penetration to lower leaves, and greater solar heating of the canopy.

The adaptive advantage of higher water use efficiency is probably greatest in hot arid zones where the combination of high temperature and low rainfall can preclude sufficient net primary productivity by most C₃ species (Schulze et al., 1996). Consistently, in the hot deserts of the world (in Somalia, Namibia, the Punjab of India, Northern Australia, and southwestern North America) grass and sedge floras are over 90% C₄, and large numbers of C₄ dicots are found (Sage et al., 1999b). Similarly, C₄ floristic representation is very high on arid tropical
islands. For example, on the Galapagos Islands, the grass flora is over 90% C₄ and C₃ dicots are common as well (Wiggens and Porter, 1971). In addition, in warm temperate deserts, summer active herbaceous species are primarily C₄, in contrast to winter active species that are almost all C₃ (Shreve and Wiggens, 1964; Smith and Nobel, 1986). The high proportion of C₄ herbs in these deserts may result in part from a failure of C₃ herbs to establish under the harsh conditions that often include soil temperatures above 50 °C and relative humidity below 10%. Whereas the high WUE of C₄ species may allow for substantial carbon gain under the very high evaporative demands of these regions, the low WUE of C₃ species may prevent positive carbon gain except at dangerously high rates of transpiration (Schulze et al., 1996).

These differences in WUE may be most significant during the seedling stage, when plants live in the hot boundary layer of the soil yet do not have the root volume required to supply the necessary amounts of water for sustained C₃ photosynthesis. While enhanced water use efficiency has apparent value in specific instances, it will not guarantee C₄ success in dry environments. Similarly, arid conditions are not required for C₄ success. Cold, dry environments such as polar or alpine deserts lack a C₄ flora, and cold deserts of western North America and Northern Mongolia have few C₄ grasses (Sage et al., 1999b). In Mediterranean type climates of Southern Europe, North Africa, South Africa, Chile, California and Southwest Australia, C₄ plants are uncommon except as summer active weeds where irrigation provides soil moisture, or in marshes. Mediterranean zones are characterized as having wet winters and dry summers. Although summer days are commonly hot, the lack of summer precipitation prevents the establishment of a native C₃ flora in most Mediterranean-type habitats. Mediterranean zones now have a rich exotic C₄ flora, however, due largely to agricultural irrigation which provides the necessary summer moisture.

In contrast to Mediterranean zones, monsoon regions often have relatively dry winters and wet summers. In these regions, C₄ plants predominate so long as there is not an established canopy of woody C₃ species. Thus, monsoon grasslands and grass areas of savannas are heavily dominated by C₄ graminoids (Solbrig, 1996; Sage et al., 1999b).

The role of summer precipitation can be seen in a comparison of climate diagrams from southern South Africa (Fig. 9). The climate around Capetown is Mediterranean, and the grass flora is >90% C₃, Durban, South Africa occurs at a similar latitude but in a monsoon zone where 70% of the annual precipitation occurs during the warm season and >75% of the regional grass flora is C₄ (Fig. 9; Vogel et al., 1978; Ellis et al., 1980). In South Africa, the best predictor for C₄ occurrence is not total precipitation but the proportion of precipitation that falls during the warm season. Where the warm season precipitation is proportionally high, C₄ grasses dominate the grass flora (Vogel et al., 1978). Similarly, in the Great Plains of North America, regional dominance by C₄ grasses rises with increasing summer precipitation (Epstein et al., 1997).

Stowe and Teeri (1978) reported high correlation between measures of aridity and floristic occurrence of C₄ dicots. This work has been cited frequently in support of the hypothesis that C₄ photosynthesis is an adaptation to aridity. The high correlation Stowe and Teeri report, however, may be an anomaly arising from changes in life form in different communities. For example, many of the C₄ species in Arizona are summer-active annuals that germinate in response to summer rains, and are not necessarily drought-adapted because they complete their life cycles before experiencing severe moisture deficiency.

Numerous studies of local distributions of arid zone species show little relationship between photosynthetic pathway and occurrence along gradients of moisture availability. In Arizona, the C₃ grass Stipa neomexicana occupied dry ridge-top sites whereas the lower topographic positions with more soil moisture were occupied by C₄ species (Gurevich, 1986). Along local topographic gradients in the Nebraska Sand Hills, C₃ species are most abundant on swales between sandy ridges (Barnes and Harrison, 1982). Soils here are finer than on the ridges, and hold significantly more moisture in the early spring to the benefit of the C₃ grasses. By early-to-mid summer, this moisture becomes exhausted, to the detriment of the C₄ species. C₄ species are more abundant on the sandy ridge-tops, where moisture remains available during the summer. While soil properties rather than photosynthetic performance may be the more immediate cause of the ecological success on these soils, these patterns ultimately arise from physiological controls that reflect interactions between the timing of water availability, differences in phenology and photosynthetic performance of C₃ and C₄ species.

A clear example that aridity is not a prerequisite for C₄ success is the dominance of flooded savannas, riverbanks and lake margins in tropical regions by C₄
grasslands and wetlands. Along floodplains of the Amazon, the C₄ grass *Echinochloa polystachya* forms dominant stands that establish on exposed riverbanks when the river is low, and form monocultures that grow as fast as the river rises, eventually forming dense mats of river vegetation (Junk, 1983; Piedade et al, 1994). Similarly, in Africa, the sedge *Cyperus papyrus* (papyrus), forms dense mats along river and lake shallows, where drought is rarely a factor (Jones, 1986). There is obviously no need to conserve water in these flooded settings. Instead, the ability of these species to attain high photosynthetic and productivity rates under warm to hot conditions is more critical. An important feature of plants in flooded locations is the ability to become established during low water, and then outgrow the rising waters following the start of the wet season. Only through C₄ photosynthesis does the ability to exhibit the necessarily high growth rates appear to be realized (Long, 1999).

### 3. The Importance of Ecological Disturbance in Arid Climates

The association of C₄ species with aridity also arises because of a number of indirect factors related to disturbance events such as fire and grazing. Aridity promotes fire that suppresses C₃ woody competitors, therefore favoring C₄ grasses. Also, the slower growth of woody vegetation in arid zones prolongs the period where it is vulnerable to disturbance events (Goldammer, 1993; Bond and van Wilgen, 1996). If disturbance is prevented, for example, by reducing populations of large herbivores such as elephants and giraffes, or suppressing fire, then many, if not most, C₃-dominated ecosystems will shift to C₄-dominated shrublands or forest. Exceptions to this pattern occur in saline areas, seasonally flooded soils, hot deserts, and possibly soils with very low fertility, where fire frequencies are already low.

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**B. Salinity**

Plant communities of saline habitats are known to have a relatively high proportion of C₄ species. Large areas of temperate coastal salt marshes are often dominated by C₄ grasses in *Spartina, Distichlis, Sporobolus* and *Zoysia* (Archibold, 1995) while saline and alkaline soils in deserts are rich with C₄ members of the family Chenopodiaceae (Walter and Box, 1983a,b). In salt marshes, C₄ species are able to maintain dominance into much cooler climatic settings than is the case on adjoining non-saline soils (Long, 1983; Guy et al., 1986). In the cold deserts of North America, lowland saline soils dominated by the C₄ species *Atriplex confertifolia* and *Distichlis*...
spicata give way to well-drained alluvial soils dominated by C₃ species such as Artemesia tridentata (West, 1983). C₄ halophytes are common as high as 50°N in Atlantic coastal marshes of eastern Canada and the British Isles, yet immediately inland at latitudes above 45°N, C₄ species are rare (Sage et al., 1999b).

Despite this common occurrence of C₄ plants in saline conditions, the functional reasons for this association, if any, have yet to be confirmed. C₄ plants have an obligate requirement for trace amounts of Na⁺ (Brownell and Crossland, 1972) but the concentration needed to meet this requirement is present in nearly all terrestrial habitats. Halophytes survive in saline habitats mostly because of a) selective exclusion of salts at the roots, b) partitioning of salt that does enter the transpiration stream into storage vacuoles, salt glands or salt bladders, and c) because they are able to osmotically adjust to the low water potentials (Flowers et al., 1977; Larcher, 1995). C₄ photosynthesis plays little direct role in these processes. Consistently, the most salt tolerant plants along salinity gradients are succulent, C₃ halophytes such as Sarcobatus and Salicornia spp. In saltmarshes where C₄ grasses often form pure stands, C₄ photosynthesis is most important in zones of intermediate salinity (MacDonald, 1977; Pomeroy and Wiegert, 1983). Here, the success of C₄ species has been hypothesized to result from the inherently greater WUE, which would reduce the water flux through the plant per unit of growth and therefore reduce the amount of salt that must be screened, extruded, stored, or secreted (Osmond et al., 1982; Adam, 1990). For C₄ species, this possibility has not yet been experimentally confirmed. Evidence from comparative studies of C₃ and C₄ salt-marsh halophytes show slightly greater performance of photosynthesis in C₄ Spartina species at intermediate salinities than C₃ Scirpus spp which often occurs nearby (Pearcy and Ustin, 1984); others show no difference (Dejong et al., 1982).

Ironically, the best evidence that high WUE is important to success in saline areas comes from mangroves, which are all woody and all C₃. In tropical regions, C₃ grasses, sedges and dicots are well represented in saline areas, and tend to dominate early succession stages in coastal salt marshes (MacDonald, 1977; Costa and Davy, 1992). Eventually mangroves invade and convert the salt marsh into a mangrove swamp. As with inland forests, arborescence confers ecological advantage to the C₃ species. Salt tolerance in mangroves has been extensively studied (Ball, 1988), and provides a model to explain why C₄ plants are successful in saline areas. In mangroves, water use efficiency is very high for C₃ species because of conservative stomatal behavior and leaf orientations that minimize heat loading. Stomata show pronounced midday closure, and will close under high evaporative demand and during high transient salinity (Ball and Farquhar, 1984; Ball et al., 1988). Only during cooler periods of greater relative humidity, when evaporative demand is lowest, will stomata reach maximum apertures. By minimizing transpiration in this manner, salt uptake via the transpiration stream is minimized. If the plants then grow fast enough so that the salt that is accumulated is sequestered into new storage tissues and vacuoles, accumulation of salt to lethal levels can be prevented. Similarly, in C₄ plants, the inherently high water use efficiency should allow for relatively low salt loading at the roots, while the high photosynthetic capacity at low CO₂ should allow for high productivity (Adam, 1990).

For unknown reasons, mangroves fail to colonize temperate zones (MacDonald, 1977). Perhaps, low temperature impairs their salt exclusion mechanisms. In any case, where mangroves cannot grow, C₄ plants dominate salt marshes up to latitudes where the growing season is too cold, at which point C₃ graminoids such as Puccinellia dominate the marshes (Archibold, 1995). This gives rise to the phenomenon where C₃ mangroves dominate coastal estuaries in tropical and subtropical zones, C₄ graminoids dominate estuaries in temperature zones where mid-winter cold excludes the mangroves, and C₃ graminoids dominate the estuaries where mid-summer cold excludes the C₄ species. Identifying the mechanisms governing this biogeographical pattern will be one of the important contributions to ecophysiology in the next century.

C. Mineral Nutrition

1. Physiology

An important advantage of the C₄ relative to the C₃ pathway is that it is less expensive to terms of nitrogen and other mineral resources invested in the photosynthetic machinery (Brown, 1977). The more efficient use of N in C₄ than C₃ photosynthesis has three primary causes. First, the maintenance of high CO₂ in the bundle sheath means that C₄ Rubisco
operates near its $V_{\text{max}}$, in contrast to C$_3$ plants where the enzyme operates below the $K_m$. Thus, for a given photosynthetic rate above 20 °C, C$_4$ species require only 20% to 40% of the Rubisco that is required by C$_3$ species. Second, the suppression of photorespiration in C$_4$ plants leads to a further increase in the efficiency of Rubisco use. Third, the form of Rubisco expressed by C$_4$ species has on average, a 20% to 30% higher turnover capacity, so that less of the enzyme is required to support a given photosynthetic rate (Seemann et al., 1984; Sage and Seemann, 1992). In agreement with the theoretical predictions, C$_3$ species have three to four times as much Rubisco on average as C$_4$ species with equivalent assimilation rates (Table 3; Sage et al., 1987; Sage and Seemann, 1993). Because Rubisco is large (550 kilodaltons), and relatively inefficient (with a maximum specific activity that is 10% of PEPCase), the enzyme is an expensive sink in terms of nitrogen. C$_4$ species maintain 5% to 10% of their total leaf nitrogen in Rubisco, while C$_3$ species maintain 15% (shade plants) to over 30% (crops) of leaf nitrogen in Rubisco (Sage et al., 1987; Evans, 1989). C$_4$ pump enzymes represent a N cost to C$_4$ photosynthesis not shared by C$_3$ species but this is relatively low and is offset somewhat by the N cost associated with the photorespiratory pathway in C$_3$ species. For example, in *Amaranthus retroflexus* (C$_4$), PEPCase contains about 5% of total leaf N, which combined with Rubisco accounts for a total of 14% of the leaf N in this species (Table 3). By contrast, in *Chenopodium album* (C$_3$), about 1.5 times N is invested in carboxylating enzymes at equivalent leaf N levels. At equivalent CO$_2$ assimilation rates, the N cost of carboxylating enzymes rises to be over 2.5 times as much N in the C$_3$ species than the C$_4$.

Nitrogen use efficiency (NUE) can be expressed in a variety of ways such as the biomass produced per unit of nitrogen, harvest yield per unit nitrogen input, instantaneous photosynthetic rate per unit leaf nitrogen, and the slope of the relationship between photosynthetic capacity and leaf nitrogen content. The slope of net CO$_2$ assimilation versus leaf nitrogen content is one of the more robust expressions of C$_3$ and C$_4$ differences in NUE because it most closely reflects the consequence of the differences between C$_3$ and C$_4$ photosynthetic physiology. At moderate temperature (20 to 27°), the A/N slope is 50% greater in C$_4$ than C$_3$ species, but it is over 100% greater at warmer temperature (34 °C) (Table 4). Differences in NUE are most pronounced at high N levels, but at high N, C$_3$ species often allocate more nitrogen to leaves, thereby compensating for the greater NUE of the C$_4$ species. For example, *Chenopodium album* (C$_3$) plants grown at high N had 30% more N per unit leaf area than *Amaranthus retroflexus* (C$_4$) plants grown at the same N levels (Sage and Pearcy, 1987a). Similarly, Li (1993) observed that the maximum leaf

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**Table 3. The content of carboxylating enzymes in the C$_3$ plant *Chenopodium album* and C$_4$ *Amaranthus retroflexus*, expressed on an absolute basis, as a ratio between the species, and as a percent of total leaf nitrogen (in parentheses).** Leaf nitrogen (N) contents were selected to represent ratios at equivalent leaf N, and at equivalent CO$_2$ assimilation capacities. Data were estimated from regression equations of net CO$_2$ assimilation versus leaf N, Rubisco content versus leaf N, and total carboxylase content versus leaf N. From Sage et al. (1987).

<table>
<thead>
<tr>
<th>Leaf Nitrogen Content$^a$, mmol m$^{-2}$</th>
<th>160</th>
<th>215</th>
<th>215/160</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_3$ rate of CO$_2$ uptake$^b$</td>
<td>29</td>
<td>45</td>
<td>—</td>
</tr>
<tr>
<td>C$_4$ rate of CO$_2$ uptake</td>
<td>47</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C$_3$ Rubisco content</td>
<td>3.3 (24%)</td>
<td>4.9 (26%)</td>
<td>—</td>
</tr>
<tr>
<td>C$_4$ Rubisco content</td>
<td>1.3 (9%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C$_3$/C$_4$ Rubisco Ratio</td>
<td>2.4</td>
<td>—</td>
<td>3.7</td>
</tr>
<tr>
<td>C$_3$ Carboxylase content$^c$</td>
<td>3.3 (24%)</td>
<td>4.9 (26%)</td>
<td>—</td>
</tr>
<tr>
<td>C$_4$ Carboxylase content</td>
<td>1.9 (14%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C$_3$/C$_4$ Carboxylase ratio</td>
<td>1.5</td>
<td>—</td>
<td>2.4</td>
</tr>
</tbody>
</table>

$^a$ *A. retroflexus* did not exhibit leaf N levels above 170 mmol m$^{-2}$.

$^b$ Units: Net CO$_2$ uptake rate in $\mu$mol m$^{-2}$ s$^{-1}$; Rubisco and total carboxylase content in g m$^{-2}$.

$^c$ Carboxylase content refers to Rubisco + PEPCase. In *C. album*, PEPCase content was insignificant compared to Rubisco content.
N of two C4 Cyperus species was 60 to 80 mmol m\(^{-2}\), while that of two C3 Cyperus spp was about 30 mmol m\(^{-2}\) higher.

2. Ecological Aspects

The high photosynthetic nitrogen use efficiency of C4 photosynthesis could be expected to provide a significant advantage under nitrogen limitation. To effectively evaluate this hypothesis, plants adapted to some level of nutrient deficiency should be compared. In plants requiring high levels of N, such as weeds and crops, photosynthetic NUE is poorly correlated with performance (Sage and Pearcy, 1997a; Long, 1999). This is not surprising, given that on N enriched soils, high capacities for leaf production and growth are more closely related to performance, while on nutrient poor soils, storage, nutrient retention, and conservative growth patterns appear to be more fit (Chapin, 1980). A higher NUE may have significance on high N soils if it enables C4 species to allocate more N to leaf area and root production, thereby giving C4 plants better access to other resources essential to growth and competitive ability. Some circumstantial evidence supports this possibility (Sage and Pearcy, 1987a; Long, 1999).

In contrast to crops and their weeds, soils of the mixed-grass prairies of central North America are commonly N deficient. The C3 grasses (Poa pratensis, Agrostis scabra, Agropyron repens) and C4 grasses (Schizachyrium scoparium, Andropogon gerardi) of these soils show responses to soil N that directly reflect differences in NUE (Wedin, 1995; Wedin and Tilman, 1993, 1996). In this ecosystem, C4 species are often dominant, particularly in late successional stages and on unfertilized soils (Wedin and Tilman, 1990). In a series of experiments designed to evaluate effects of N addition, Wedin and Tilman found that nitrogen use efficiency (plant biomass per N use) of the C4 grasses was approximately double that of the C3, and on plots receiving no N fertilizer, C4 biomass was 20% to 60% of total biomass at midsummer (Wedin and Tilman, 1996). At N fertilization rates above 10 gN m\(^{-2}\) year\(^{-1}\), the C3 grasses completely crowded out the C4 species (Wedin and Tilman, 1992, 1996). The mechanisms underlying this competitive shift are complex, and show some of the intricacies of how physiological differences give rise

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<table>
<thead>
<tr>
<th>Species</th>
<th>PNUE (\mu\text{mol CO}_2\text{ mol}^{-1}\text{N})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C4 Plants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyperus papyrus</td>
<td>280</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Cyperus longus</td>
<td>391</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Cyperus japonicus</td>
<td>380</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Echinochloa crusgalli</td>
<td>580 @ 34 °C</td>
<td>R. F. Sage unpublished</td>
</tr>
<tr>
<td>Miscanthus sinensis</td>
<td>247</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Spartina cynosuroides</td>
<td>377</td>
<td>M. B. Jones (in Li, 1993)</td>
</tr>
<tr>
<td>Amaranthus retroflexus</td>
<td>350 @ 20 °C 420 @ 27 °C 520 @ 34 °C</td>
<td>Sage and Pearcy, 1987b</td>
</tr>
<tr>
<td><strong>C3 Plants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyperus alternifolium</td>
<td>252</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Cyperus involucratus</td>
<td>256</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Cyperus vegetus</td>
<td>180</td>
<td>Li, 1993</td>
</tr>
<tr>
<td>Typha domingensis</td>
<td>53</td>
<td>M. B. Jones (in Li, 1993)</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>200 to 250 @ 25 °C</td>
<td>Makino et al., 1994; Makino et al., 1997</td>
</tr>
<tr>
<td>Solidago altissima</td>
<td>140</td>
<td>Hirose and Werger, 1987</td>
</tr>
<tr>
<td>Chenopodium album</td>
<td>230 @ 20 °C 280 @ 27 °C 240 @ 34 °C</td>
<td>Sage and Pearcy, 1987b</td>
</tr>
</tbody>
</table>
to ecological mechanisms (Wedin, 1995; Wedin and Tilman, 1992, 1996). In one of the C4 species, *Schizachyrium scoparium*, high photosynthetic production potential enabled it to maintain a higher root fraction than C3 competitors. In the absence of fertilization, this enabled the C4 grasses to reduce available soil N to levels too low for growth of the C3 plants. Second, the higher photosynthetic NUE enabled the C4 species to maintain high C:N ratios in leaves, which reduced the forage quality of the C4 grasses and thus, the probability of herbivory. In addition, high C:N in leaves gave rise to litter with high C:N, and this decomposed more slowly such that N cycling also slowed (Wedin, 1995). This further contributed to low soil N availability. Fertilization disrupted these controls, and by supporting rapid growth of the C3 species, produced a dense C3 canopy in early spring that was able to shade, and therefore suppress, the C4 biomass during the summer (Wedin, 1995; Sage et al., 1999b).

V. The Functional Role of Photosynthetic Subtype

An important advance in the 1980’s was the development of anatomical screen that segregated most (>80%) of the world’s grass genera to either the malate-forming NADP-ME subtype, or the aspartate forming NAD-ME and PCK subtypes (Hattersley, 1983; Hattersley and Watson, 1992). Further refinements in recent years have enabled segregation of many NAD-ME and PCK species into their respective sub-types (Watson and Dallwitz, 1998). As a result of these advances, most grasses have now been classified as C3 or into one of the C4 sub-types (Sage et al., 1999a).

Significant correlation is evident between C4 biochemical subtype and aridity despite there being no correlation between total C4 representation and aridity. At mesic ends of aridity gradients, NADP-ME species dominate the C4 grass flora; at xeric ends, NAD-ME species predominate (Fig. 10). These patterns have been well described for grasses from Australia (Prendergast, 1989; Henderson et al., 1994), Namibia (Schulze et al., 1996), South Africa (Ellis et al., 1980), central Asia (Pyankov and Mokronosov, 1993), and South America (Knapp and Medina, 1999). Our examination of regional North American floras indicates the correlation between photosynthetic subtype and precipitation is robust here as well.

![Fig. 10. Relationships between the percent representation of C4 subtypes in regional C4 grass floras in Australia, and the lowest median annual rainfall in the regions. (Adapted from Hattersley, 1992.)](image)

Interestingly, Pyankov and Mokronosov (1993) report a similar observation between salinity and subtype, with NAD-ME species being common on high salinity soils, while NADP-ME species are floristically much less common.

No correlation between subtype and aridity or salinity has been observed among sedges and dicots. In sedges, the NAD-ME subtype is present only in *Eleocharis*, which contains about 200 of the estimated 1500 C4 sedge species; all other sedges are NADP-ME (Sage et al., 1999a). In dicots, the taxonomic distribution of photosynthetic subtypes is too poorly known to allow meaningful assessments; however, most C4 dicots occur on arid or saline habitats, so it is likely that no pattern will arise.

These correlations indicate there is some advantage of the NAD-ME as compared to the NADP-ME decarboxylation mechanism under low rainfall conditions, but currently there is little known about the underlying mechanism (Hattersley, 1992). The advantage of the NADP-ME subtype under wetter
VI. Photosynthesis in the Future

A. Cursory Observations

Currently, human activity is restoring atmospheric CO₂ contents to levels not seen for millions of years. Theoretically, this will remove the selective pressure that may have supported C₄ evolution and radiation, leading to the possibility that C₃ vegetation will expand at the expense of C₄ vegetation, in some cases causing extinction of C₄ species. In recent years, extensive discussion and increasingly sophisticated experiments have focused on this issue. Rather than summarize the details of this work (readers are referred to Patterson and Flint, 1990; Henderson et al., 1994; Polley, 1997; Sage et al., 1999b for more detailed discussions), we highlight some key features that have arisen from discussions of C₄ and C₃ responses to future global change:

1. High CO₂ stimulates C₃ photosynthesis more than C₄ in the short term, but the ability to exploit CO₂ enrichment depends upon long-term enhancement of photosynthesis (Kirschbaum et al., 1995; Bowes, 1996). In C₃ plants, maintenance of photosynthetic stimulation by high CO₂ depends upon sustained availability of nutrients. Experiments conducted on soils with high nutrient availability show increased C₃ biomass at the expense of C₄ biomass in high CO₂ conditions (Patterson and Flint, 1990; Drake et al., 1996). With soil nutrient deficiency, a series of feedbacks at the leaf, whole plant and soil level become apparent that reduce long-term photosynthetic and growth responses of the C₃ species (Bazzaz, 1990; Stitt, 1991; Diaz et al., 1993). High CO₂ may also aggravate soil nutrient deficiency, because it promotes higher levels of soil carbon, and creates high C:N litter that decomposes more slowly (Diaz et al., 1993; Ball, 1997). Both effects lead to sequestration of nutrients into less mobile pools and a slowing of nutrient cycling. Because many C₄ grasses are favored by N deficiency (Wedin and Tilman, 1996), such effects of high CO₂ on soil fertility could promote C₄ species at the expense of C₃.

Alternatively, nitrogen eutrophication of landscapes by sewage, agricultural fertilizers, and industrial pollutants promote C₃ dominance by reducing the consequences of NUE advantages of C₄ species, and by increasing the ability of C₃ species to exploit CO₂ enrichment. Moreover, high CO₂ coupled with nitrogen (N) deposition will likely favor more rapid establishment of woody species, thereby accelerating succession from grasslands to forests (Sage et al., 1999b). Human production of N fertilizers has increased greatly in recent decades to the point where anthropogenic N fixation is approximately equal to natural N fixation (Vitousek, 1994). Much of this anthropogenic N is deposited onto terrestrial ecosystems. Because human demands for reduced N are increasing, it is certain that predictions of future C₄/C₃ vegetation dynamics will need to account for N enrichment effects.

2. High CO₂ reduces stomatal conductance in both C₃ and C₄ species, apparently by similar magnitudes (Sage, 1994; Polley, 1997). This enhances water use efficiency and leads to reductions in water consumption, delaying the onset of water stress and reducing its severity (Owensby et al., 1996, 1997). Although both C₄ and C₃ species potentially benefit from such responses, in natural ecosystems the C₃ species are more likely to benefit. The growing season of C₄ plants occurs when evaporative demand is greatest, and thus potential reductions in transpiration caused by stomatal closure are potentially more significant. Consistent with this hypothesis, exposure of North American grassland communities to double the current atmospheric CO₂ favored the C₄ grasses, largely as a result of a longer growing season in dry years and reduced inhibition from water stress (Owensby et al., 1996, 1997).
3. C₄ species often show substantial acclimation to high CO₂ that appears to be driven by carbohydrate status of the plants (Sage, 1994; Bowes, 1996). This can reduce the N cost of photosynthesis and may lead to enhanced NUE and performance in high CO₂ conditions (Sage et al., 1997). In C₄ species, acclimation to elevated CO₂ could also lead to a lower N cost of photosynthesis and increases in NUE. In high CO₂, C₄ photosynthesis operates well above the CO₂ saturation point, indicating PEPCase is nonlimiting (Sage, 1994). Theoretically, acclimation could reduce PEPCase without affecting A, and this would be observed as a reduction in the initial slope of the photosynthetic CO₂ response and a rise in the CO₂ saturation point. In reality, this does not appear to be the case, because C₄ plants grown and measured at high CO₂ show no qualitative shifts in their photosynthetic CO₂ responses (Wong, 1979; Sage, 1994). Some quantitative changes have been noted, in that modest reductions in A can occur following long term exposure to high CO₂ (Wong, 1979; Morgan et al., 1994).

4. Global warming may be expected to favor C₄ plants, given their greater response to high temperature. More important than the degree of warming, however, is likely going to be the seasonal timing of the warming. Models of climate change predict that wintertime and high latitudes will experience the greatest level of warming (Kattenberg et al., 1996). These periods are typically the time when C₃ species are active (Monson et al., 1993), so that where winters and early-spring may become milder, the ‘cool’ growing season favorable to C₄ plants may improve. Some evidence for this is present in recent data from the plains grasslands of south-central Canada where the C₄ grasses have expanded in recent decades, apparently because of milder winter temperatures and longer spring growing seasons (Peat, 1997; Skinner et al., 1998).

Similarly, the response of species to changing temperature and rising CO₂ will also depend on when precipitation falls, with the most dramatic shifts likely found at temperate latitudes where patterns of seasonal drought may be altered. If summer heating leads to more frequent and severe drought, C₄ species may become inhibited. Alternatively, where monsoon precipitation patterns become established, the C₄ species will likely be favored. One region that may be affected by monsoon intensification is the southern Great Basin desert of Nevada and Utah, where summer precipitation is currently sparse. Monsoon rains that support a rich C₄ flora in the Mojave and Sonoran deserts to the south of the Great Basin are predicted to shift northward (Lin et al., 1996), and with it, many of the southern C₄ species may become common on landscapes further north.

5. Interactions between high CO₂ and climate warming are difficult to predict because warming alone favors C₄ species while high CO₂ alone favors C₃ species, particularly at warmer temperatures. Studies of interactive effects of temperature and CO₂ show that high CO₂ enables the C₃ species to narrow the gap in photosynthesis and growth between the C₃ and C₄ species in warmer temperatures but does not completely overcome the differences (Hunt et al., 1996; Grise, 1996). Part of the reason why C₄ species remain superior at warmer temperatures is because they have evolved many features that promote fitness in warm habitats. For example, allocation strategies, phenology of growth and reproduction, heat tolerance, and thermal optima of cellular processes are some of the non-photosynthetic parameters that can be critical to survival in hot environments. Because many potential C₃ competitors are adapted to habitats and seasons of lower temperature, they may remain unfit despite improved photosynthetic performance in high CO₂ (Bazzaz, 1990).

C. Paleoecological Perspectives

One of the best approaches to assessing the future of C₄ photosynthesis is to use the tools of paleoecology to evaluate how past episodes of CO₂ and climate change affected the distribution of C₄ relative to C₃ species. This has already provided some valuable insights.

1. Theoretical Issues

At the height of the last ice age 21,000 years ago, atmospheric CO₂ content was 180 μmol mol⁻¹ and mean global temperature about 7 °C cooler relative to today (Barnola et al., 1987; Kutzbach et al., 1998). Between 15,000 and 10,000 years ago, CO₂ levels rose from 180 μmol mol⁻¹ to the Holocene average of...
270 \, \mu\text{mol mo}^{-1} \, (\text{Fig. 1C}). Temperature also rose during this period, eventually peaking about 8,000 years ago during the Holocene Hypsithermal episode.

Theoretically, these changes should have profound effects on C\textsubscript{3} and C\textsubscript{4} vegetation dynamics. Reducing CO\textsubscript{2} levels to 180 \, \mu\text{mol mo}^{-1} substantially reduces photosynthetic carbon gain of C\textsubscript{3} species but causes only marginal photosynthetic reductions in C\textsubscript{4} species (Sage, 1995). At the CO\textsubscript{2} levels of the last ice age and at temperatures of 25 °C to 30 °C, photosynthesis is approximately double in C\textsubscript{4} relative to functionally similar C\textsubscript{3} plants (Fig. 3; 7; Tissue et al., 1995). With respect to growth, \textit{Amaranthus retroflexus} (C\textsubscript{4}) produces 23 times more biomass than the ecologically similar weed \textit{Abutilon theophrasti} (C\textsubscript{3}) at 150 \, \mu\text{mol mo}^{-1} and 28 °C, but only 2.6 times more biomass at 270 \, \mu\text{mol mo}^{-1} (Dippery et al., 1995). At 350 \, \mu\text{mol mo}^{-1}, the two species are equivalent in biomass production. In soil plots extracted from Texas prairies, increasing growth CO\textsubscript{2} concentration from 200 to 270 \, \mu\text{mol mo}^{-1} reduced frequency of C\textsubscript{4} vegetation by 37% and the C\textsubscript{4}/C\textsubscript{3} biomass ratio by 78% (estimated from Johnson et al., 1993). These responses indicate C\textsubscript{4} plants could have expanded across landscapes during low CO\textsubscript{2} intervals of the Pleistocene. Not only would warm habitats have been affected, because C\textsubscript{4} species would theoretically become more competitive in cool temperate settings they do not currently dominate (Jolly and Haxeltine, 1997). However, the cooler global temperatures of the last glacial maximum would have partially offset the effect of low CO\textsubscript{2}, which combined with other ecological factors could have attenuated shifts in C\textsubscript{4} distributions (Collatz et al., 1998).

2. Empirical Observations

Consistent with the theoretical predictions, paleoecology studies indicate increased C\textsubscript{4} grass abundance during the low CO\textsubscript{2} episodes of the last glacial maximum; however, this expansion is not global, and may be related to increased aridity in addition to, or instead of, low CO\textsubscript{2}. Relative to the current era, central Africa experienced widespread expansion of C\textsubscript{4} grasslands during the last glacial maximum 15 to 25 kYA. Both upland and lowland sites from Ghana in West Africa to Kenya in East Africa show increases in grass and sedge pollen (most likely C\textsubscript{4} species in these latitudes) (Maley, 1991; Sowunmi, 1991; Aucour et al., 1994; Giresse et al., 1994; Street-Perot, 1994). Correspondingly, carbon isotope ratios from central-African lakes are significantly more positive in late Pleistocene than early Holocene sediments that are 12,000 to 8,000 years old (Hillaire-Marcel et al., 1989; Ehleringer et al., 1997; Street-Perot et al., 1997). At these sites, the observed shifts in carbon isotope ratio from C\textsubscript{4} to C\textsubscript{3} like-values correspond to the 30% increase in CO\textsubscript{2} at the end of the Pleistocene. This correlation is interpreted as evidence for a CO\textsubscript{2}-control over C\textsubscript{4} distribution, with the rise in CO\textsubscript{2} causing, in part, the contraction of C\textsubscript{4}-dominated grasslands (Ehleringer et al., 1997; Street-Perot et al., 1997).

While consistent with theoretical predictions, these studies must be interpreted with caution for a number of reasons. First, drier conditions are apparent over much of central Africa at the last glacial maximum (Street-Perot, 1994; Jolly et al., 1998) and this would have strong influences over the distribution of grasslands and forest regardless of CO\textsubscript{2} level. Effects of aridity versus CO\textsubscript{2} change remain to be disentangled. Second, in some of the studies where C\textsubscript{4} grass retreat is evident 10,000 to 15,000 years ago, the cores extend to deposits older than 30,000 years. Between 25,000 and 35,000 years old, CO\textsubscript{2} levels remained below 200 \, \mu\text{mol mo}^{-1}, yet some of the cores of this age show strong shifts in carbon isotope ratios from values indicative of C\textsubscript{3} vegetation (Talbot and Johannesson, 1992; Aucour et al., 1994; Street-Perot, 1994). Third, the African trends are not apparent for many areas of the planet. In Kashmir, India, and western Java, for example, isotopic change indicates shifts from C\textsubscript{4} to C\textsubscript{3} vegetation during the low CO\textsubscript{2} levels of the late Pleistocene (Krishnamurthy et al., 1982; Kaars and Ram, 1997). Pollen evidence also indicates much of the tropical South America was woodland at the end of the Pleistocene (Bush et al., 1990; Colinvaux, 1993). However, consistent with the results from Africa, savanna-type vegetation appears to have expanded into rainforest over parts of Amazonia and gallery forest in southeastern Brazil during the period corresponding to the end-Pleistocene CO\textsubscript{2} low (van der Hamer and Apsy, 1994; Behling and Lichte, 1997). As in Africa, this expansion is also associated with greater aridity in this region at this time (van der Hamer and Apsy, 1994; Behling and Lichte, 1997).

In the tropics, life form differences often dominate influences of photosynthetic pathway to favor woody C\textsubscript{4} species over herbaceous C\textsubscript{3} species. It is possible that the failure to see an expansion of C\textsubscript{4} vegetation in many areas reflects the persistent dominance of
the woody biota at low CO$_2$. To more precisely evaluate how C$_3$ and C$_4$ vegetation respond to past change in climate and CO$_2$, it may be better to examine responses in landscapes where C$_3$ and C$_4$ vegetation of similar life form occur, namely the steppes and grasslands of North America, Asia, and temperate Africa.

In the temperate zones, pollen evidence shows no consistent correlation between possible C$_4$ vegetation occurrence and the CO$_2$ rise 15,000 to 10,000 years ago (Villagram and Armento, 1993; Fredlund, 1995; Sun et al., 1997). It is difficult, however, to detect the presence of C$_4$ species from pollen evidence alone, so these results cannot be considered definitive and carbon isotopic evidence is necessary to compliment pollen and plant macrofossil evidence. In contrast to predictions, however, carbon isotope data don’t show obvious signs of enhanced C$_4$ dominance during the last glacial maximum. In temperate South Africa and east-central China, no strong shift between C$_3$ and C$_4$ dominance are apparent in carbon isotope records from deposits 10,000 to 27,000 years old (Lee-Thorp and Beumont, 1995; Wang et al., 1997). A number of detailed studies from the southwestern U.S. also show no consistent trend. In eastern New Mexico, carbon isotope ratios indicate enhanced C$_4$ dominance at the last glacial maximum, with C$_3$ shrubs encroaching into C$_4$ grasslands about 8,000 years ago (Cole and Monger, 1994). Cole and Monger interpret this shift as a response to the CO$_2$ rise of a few thousand years earlier, although this interpretation has been questioned (Boutton et al., 1994). In a study of carbon isotopes from tooth enamels of ice-age grazers (Mammuthus, Bison, Equus and Camelops spp) of New Mexico and Arizona, Connin et al. (1998) inferred that regional patterns of C$_4$ abundance at the late-Pleistocene changed little in the Holocene, despite the changes in CO$_2$ and temperature. As today, C$_4$ grasses were abundant in southeast Arizona and southern New Mexico, but uncommon further north and west. While this lack of change in C$_4$ distribution could result from antagonistic effects of rising CO$_2$ and temperature, Connin et al. (1998) note that summer rainfall during the Pleistocene may have been the critical control over C$_4$ success during the ice age.

In north-central Texas, Humphrey and Ferring (1994) observed reductions in carbon isotope ratios in soil deposits that are 14,000 to 10,000 years old, indicating contraction of C$_4$ biomass. Further west, in central Texas, soil organic matter show increases in carbon isotope ratio between 15,000 and 8,000 years ago, indicating C$_4$ expansion in the Holocene (Nordt et al., 1994). In the southern high plains of west Texas, a similar C$_3$ to C$_4$ transition is observed, based on changes in carbon isotope ratio of Bison collagen (Stafford et al., 1994, cited in Connin, 1998). A survey of carbon in grass phytoliths from the northern Great Plains indicates increased C$_4$ grass expansion into C$_3$ grasslands of North America at the termination of the last ice age (Kelly et al., 1991), which is consistent with increasing temperature favoring the expansion of C$_4$ vegetation. One additional shift from C$_4$ to a C$_3$-enriched flora has been reported in the desert southwest of North America. Here, a desert of C$_3$ thornscrub and CAM succulents replaced a C$_4$ grassland as conditions warmed and became more arid between the last glacial maximum and the Holocene (Liu et al., 1996).

In summary, the evidence to date is consistent with the hypothesis that low CO$_2$ levels of the late Pleistocene favored more extensive C$_4$ grasslands in tropical areas; however, C$_3$ vegetation remained common and was often dominant in the tropics, particularly outside of central African locations. In temperate zones, there is no consistent evidence for a C$_3$ expansion as a result of higher CO$_2$, although there are a number of studies that indicate the warming of the early to mid Holocene period between 5,000 and 10,000 years ago promoted greater C$_4$ dominance. Taken together, these results indicate that higher CO$_2$ can promote C$_3$ species over C$_4$, but mainly in tropical areas, and mainly by promoting woody vegetation over graminoids. As is the case today, the timing of precipitation may be one of the most important determinants of the abundance of C$_4$ species in coming centuries.

VII. Conclusion: C$_4$ Plants and the Human Factor

To a certain extent, discussions of the response of natural vegetation to climate change are academic, because the rising human population is becoming so pervasive across the face of the earth and so demanding of resources that only marginal land will not be subjected to extensive human management. With regards to the future of C$_4$ vegetation, recent history serves as a pretty good guide to the probable trends that will occur as a result of increasing human dominance of ecosystem processes. The strength of
human controls is so great that trends already observed will likely overwhelm effects of any C₄ response to climate and atmospheric change.

In recent decades, humans have greatly altered the distribution of C₃ and C₄ species on the planet through heavy logging, landscape conversion to agriculture, burning, fire suppression, establishment of timber plantations, overhunting of large herbivores, and overgrazing. In the tropics, natural forest landscapes have been greatly reduced in scope by conversion to pasture, croplands or exotic grasslands (Myers, 1991; D’Antonio and Vitousek, 1992). Dry tropical forests have been severely affected, with over 80% of the natural forest area lost (Maas, 1995). Wet tropical forests are going rapidly and only remnants are predicted to exist in a few decades, with C₄ grasses occupying much of the former forested landscape (Myers, 1991). In West Africa, for example, human exploitation of landscapes has resulted in a 500 km extension of C₄ grasslands and savanna into forested regions by 1983 (Hopkins, 1983). These changes represent the largest expansion of C₄ biomass since the mid-Holocene, and have occurred during substantial increases in atmospheric CO₂ and nitrogen deposition.

Human action does not simply favor C₄ grasses, however. Savanna grasslands of tropical regions are increasingly being converted to timber plantations (Soares, 1990). In temperate regions, widespread loss of native C₃ grasslands has resulted because overgrazing has led to shrubland encroachment, while fire suppression and timber plantations have generated woodlands in large areas of what were once C₃ grasslands and savannas (Sage et al., 1999b). In some cases, C₃-dominated biomes have been largely eliminated, as is demonstrated by the conversion of the eastern portion of the tall-grass prairie of North America into row crop agriculture. C₃ plants are common in this former grassland, but they are mainly maize or one of its weeds.

From these observations, what appears clear is that the future of C₄ plants will largely depend upon human decisions concerning how the landscape will be managed. Where C₄ plants fit into the management scheme, or, as weeds, are able to exploit the management scheme, they will do extremely well. However, if they are of little use to humans, then it is possible they could, like much of the rest of the biota, be marginalized in the future, human-dominated world.

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Chapter 22

CO₂ Assimilation in C₃-C₄ Intermediate Plants

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Summary

The mode of CO₂ assimilation known as C₃-C₄ intermediate photosynthesis provides intriguing insight into a novel mechanism for reducing photorespiratory CO₂ loss and possible evolutionary pathways to C₄ photosynthesis. Plants with the C₃-C₄ pathway have been reported from twenty-five species in nine genera representing six families, and they are principally associated with warm or hot habitats. The ecological distribution of C₃-C₄ plants is consistent with an adaptive role for re-assimilation of photorespired CO₂ at warm temperatures. Photorespired CO₂ is re-assimilated through the differential partitioning of photorespiratory organelles between mesophyll cells and bundle-sheath cells in C₃-C₄ intermediates, including total isolation of glycine decarboxylase activity to the bundle-sheath. Thus, glycine diffuses to the bundle sheath tissue, where photorespired CO₂ is released and assimilated by surrounding chloroplasts before it can escape the leaf. Models of this ‘glycine shuttle’ reveal possible advantages in terms of CO₂ assimilation rate, water-use efficiency, and nitrogen-use efficiency, though there are strong constraints on the fraction of RuBP carboxylation capacity that can be allocated to the bundle sheath to assimilate the photorespired CO₂. The models further predict distinctive gas-exchange patterns with respect to the CO₂ compensation point and discrimination against ¹³CO₂. These predictions have been validated with gas-exchange measurements. One group of C₃-C₄ species (those belonging to the genus Flaveria) exhibit evidence of functional C₄ biochemistry and assimilation of at least some atmospheric CO₂ through the C₄ pathway. In the Flaveria intermediates, photorespiration rates are...
reduced as in C₃-C₄ intermediates from other genera, and in several *Flaveria* species O₂ inhibition of photosynthesis is reduced, suggesting the presence of a CO₂-concentrating mechanism. It is not understood how photorespiration rates are reduced in these ‘biochemical intermediates,’ though there is evidence that at least part of it is due to the same glycine shuttle found in other C₃-C₄ intermediates. The glycine shuttle may represent an initial step in the evolutionary path to C₄ photosynthesis.

I. Introduction

Plants that have been classified as C₃-C₄, or C₃-C₄ intermediates, exhibit a unique mode of photorespiratory compartmentation between mesophyll and bundle-sheath cells. This unique mode of photorespiration is common to all C₃-C₄ plants so far examined. It is important to realize from the outset, that the unique gas-exchange traits used to classify plants as C₃-C₄ (i.e., low CO₂ compensation point and curvilinear response of the CO₂ compensation point to O₂) are due to unique aspects of photorespiratory compartmentation, not changes in photosynthetic metabolism (as occurs in C₄ plants). Most of the C₃-C₄ intermediates have photosynthetic metabolism that is similar to C₃ plants. In a couple of genera, C₅-type photosynthesis can be found, but its role in producing the unique gas-exchange traits that typify C₃-C₄ plants is uncertain. In fact, the presence of C₄ photosynthesis in these species does not need to be invoked to explain the presence of C₃-C₄ intermediate traits. Given that photorespiratory processes underlie C₃-C₄ intermediate traits, it is somewhat misleading that this type of CO₂ assimilation is commonly labeled C₃-C₄ photosynthesis or C₃-C₄ intermediate photosynthesis. C₃-C₄ plants were first described, albeit with naiveté, in a report of anatomical variations among plants thought to possess C₄ photosynthesis (Laetsch, 1971). Among these observations was the report that the bundle sheath cells of *Mollugo verticillata*, a plant with C₅-like carbon isotope ratios, contained numerous mitochondria and chloroplasts arranged in a tight centripetal pattern, with mitochondria lining the innermost tangential wall, surrounded by chloroplasts. A few years later, Kennedy and Laetsch (1974) reported C₄-like CO₂ compensation points in this otherwise C₃-like species. *Mollugo verticillata* became the first reported C₃-C₄ species. Since that time, a number of important discoveries have been made concerning the mechanism of C₅-C₄ metabolism, the basis for C₃-C₄ gas-exchange patterns, the nature of genetic control over C₃-C₄ traits, and the significance of C₃-C₄ intermediates to the evolution of C₄ photosynthesis (for reviews see, Monson et al., 1984; Edwards and Ku, 1987; Monson and Moore, 1989; Brown and Bouton, 1993; Rawsthorne and Bauwe, 1997). In this chapter we focus on the metabolic and physiological basis for C₃-C₄ traits.

II. The Distribution of C₃-C₄ Intermediates and the Advantages of CO₂ Assimilation in C₃-C₄ Plants

C₃-C₄ intermediates have been reported in twenty-five species from nine genera representing six families (Rawsthorne and Bauwe, 1997). Many of the genera occur in families that also have fully-expressed C₄ species, although this is not universal (e.g., *Moricandia* and *Parthenium* have C₃-C₄ intermediates, but no C₄ species). Arguments have been posed to explain C₃-C₄ intermediates as evolutionary precursors to C₄ photosynthesis (Monson et al., 1984; Brown and Hattersley, 1989; Monson, 1989a), although alternative explanations are possible (Monson and Moore, 1989). Recent studies using formal phylogenetic mapping on the basis of morphological traits (Monson, 1996) and molecular markers (Kopriva et al., 1996) have solidified the conclusion that C₃-C₄ intermediate traits are the evolutionary antecedents to C₄ photosynthesis, at least in the genus *Flaveria*.

When grouped according to growth habit and ecological relationships, C₃-C₄ intermediate traits tend to occur in fast-growing herbaceous species, that often exhibit the ruderal ecological habit, and undergo their greatest growth rates during the warmest part of the growing season (Monson, 1989a). The existence of these ecological patterns has been used to argue that the C₃-C₄ mode of CO₂ assimilation provides adaptive advantages to carbon gain in warm
environments and in plants that exhibit high relative growth rates which must be accommodated by rapid rates of CO\textsubscript{2} assimilation (Monson, 1989a; Schuster and Monson, 1990). At warm temperatures photosynthesis rate reaches a maximum due to decreases in the CO\textsubscript{2}/O\textsubscript{2} solubility ratio and decreases in the affinity of Rubisco for CO\textsubscript{2} relative to O\textsubscript{2} (Ogren, 1984). Given that CO\textsubscript{2} assimilation through the C\textsubscript{3}-C\textsubscript{4} intermediate path is actually a modification of photorespiratory patterns, the advantages of this metabolic mode should be greatest at warm leaf temperatures.

III. Photorespiratory Metabolism and Compartmentation in C\textsubscript{3} Versus C\textsubscript{3}-C\textsubscript{4} Intermediate Species

A. Anatomy and Ultrastructure

The C\textsubscript{3}-C\textsubscript{4} intermediate character is determined by anatomical and biochemical components which, when integrated, give rise to the gas exchange phenotypes that are described below. The leaves of C\textsubscript{3}-C\textsubscript{4} intermediate species have a distinctive anatomy which differentiates them from either C\textsubscript{3} or C\textsubscript{4} species (Fig. 1) (Brown and Hattersley, 1989). The vascular bundles are surrounded by chlorenchymatous bundle-sheath cells which are reminiscent of the pronounced Kranz anatomy of leaves of C\textsubscript{4} plants. In C\textsubscript{3}-C\textsubscript{4} intermediates, however, mesophyll cells are not arranged in a concentric ring around the bundle-sheath as in a C\textsubscript{4} leaf (Fig. 1). Instead, the mesophyll cells are arranged as in leaves of C\textsubscript{3} species with greater interveinal distances than in the leaves of C\textsubscript{4} plants (Brown and Hattersley, 1989). Bundle-sheath cells in the leaves of C\textsubscript{3} species in the genera which include C\textsubscript{3}-C\textsubscript{4} intermediate species are chlorenchymatous but do not have specialized development that would differentiate them from the surrounding mesophyll cells (Brown and Hattersley, 1989; S. Rawsthorne, unpublished observations).

In all intermediate species studied to date, the bundle-sheath cells contain large numbers of organelles. Numerous mitochondria, peroxisomes, and many of the chloroplasts are located centripetally in the bundle-sheath cells (Edwards and Ku, 1987). Mitochondria are found along the cell wall adjacent to the vascular tissue and are overlain by chloroplasts (Fig. 2). Quantitative studies have shown that the mitochondria and peroxisomes are four times more abundant per unit cell area than in adjacent mesophyll cells and that these mitochondria have twice the profile area of those in the mesophyll (Hylton et al., 1988; Brown and Hattersley, 1989). Recent work (E. L. Rylott and S. Rawsthorne, unpublished) has revealed that this anatomical development of the bundle-sheath cells in the C\textsubscript{3}-C\textsubscript{4} intermediate Moricandia arvensis occurs from a C\textsubscript{3} default state. In other words, very early in the development of C\textsubscript{3}-C\textsubscript{4} intermediate leaves the size and number of organelles in the bundle-sheath cells are comparable to those found in closely related C\textsubscript{3} species.

A further anatomical feature of leaves of C\textsubscript{3}-C\textsubscript{4} intermediate species, at least in the genus Panicum, is revealed by a study of plasmodesmatal frequency between the bundle-sheath and mesophyll cells (Brown et al., 1983). C\textsubscript{3}-C\textsubscript{4} intermediate species have a density of plasmodesmata at the bundle-
sheath/mesophyll interface which approaches that of \( C_4 \) species and is much greater than that of the \( C_3 \) species. In \( C_4 \) species, cellular anatomy is arranged so that the metabolism of the \( C_4 \) cycle occurs in adjacent bundle-sheath and mesophyll cells (Chapters 18, Furbank et al. and 19, Leegood). The exchange of metabolites between these cells occurs through diffusion down concentration gradients and is facilitated by the high density of shared plasmodesmal connections (Chapter 19, Leegood). Based upon the present model for photorespiratory metabolism in \( C_3-C_4 \) intermediate species (as discussed below), it is likely that the high density of plasmodesmata is required to facilitate metabolite exchange between cell types.

**B. Photorespiratory Metabolism and the Importance of Glycine Decarboxylase**

The leaves of \( C_3-C_4 \) intermediate species in some genera, and notably Flaveria, are known to have activities of \( C_4 \) cycle enzymes that are elevated above those in related \( C_3 \) species. Given that \( C_4 \) photosynthesis is a mechanism that reduces photorespiration, expression of this pathway in \( C_3-C_4 \) intermediate species could be a means to decrease photorespiratory \( CO_2 \) loss. The extent to which \( C_4 \) metabolism contributes to the gas exchange phenotype of \( C_3-C_4 \) intermediate species in those plants which express it, is unclear as discussed below. However, the role of cell-specific expression of glycine decarboxylase (GDC), the enzyme that catalyzes the release of \( CO_2 \) during photorespiration (Chapter 5, Douce and Heldt), in determining the \( C_3-C_4 \) intermediate gas exchange phenotype is now undisputed.

In those species that have been studied, the maximum catalytic activities for enzymes involved in the photorespiratory pathway are broadly comparable in leaves of congeneric \( C_3 \) and \( C_3-C_4 \) intermediate species. However, there are clear differences in the compartmentation of photorespiratory reactions within the leaves of \( C_3-C_4 \) intermediate species of Alternanthera, Flaveria, Moricandia, Mollugo, Panicum, and Parthenium, compared to that in \( C_3 \) species. In the leaves of \( C_3 \) species all photorespiratory enzymes are present in all of the photosynthetic cells (Hylton et al., 1988; Rawsthorne et al., 1988ab; Tobin et al., 1989). This is not the case for \( C_3-C_4 \) species. On the basis of immunogold localization studies and measurements of enzyme activity in protoplast fractions enriched in either bundle-sheath or mesophyll cells (Hylton et al., 1988; Moore et al., 1988; Rawsthorne et al., 1988b; Devi et al., 1995) GDC is confined to the bundle-sheath cells of \( C_3-C_4 \) species. Serine hydroxymethyltransferase (SHMT) activity is also enriched in bundle-sheath cells (Rawsthorne et al., 1988b). These two enzymes together catalyze the formation of serine, ammonia and \( CO_2 \) from two molecules of glycine in the mitochondria (Chapter 5, Douce and Heldt).

The early immunogold localization studies which addressed the distribution of GDC within the leaf used antibodies raised against the P protein of the complex. This protein subunit catalyses the release of \( CO_2 \) from glycine during the GDC reaction. More recent studies have shown that in the leaves of \( C_3-C_4 \) intermediate species of Panicum and Flaveria the T, H, and L proteins of the GDC complex are also confined to bundle-sheath cells (Morgan et al., 1993). In leaves of Moricandia arvensis it is surprising that these three subunit proteins are still present in the mitochondria of the mesophyll cells (Morgan et al., 1993). The L protein, dihydrolipoamide dehydrogenase, is also a subunit (known as E3) of the mitochondrial pyruvate dehydrogenase complex.
(Bourguignon et al., 1996) and its presence would therefore be expected in the mitochondria of all cells. However, studies have shown that the amount of the dihydrolipoamide dehydrogenase in the mitochondria which is not associated with GDC is small and the abundance of L protein in the mesophyll cell mitochondria of M. arvensis is not explained by this association with other multienzyme complexes (Bourguignon et al., 1988, 1996; Turner et al., 1992). Based on present knowledge, the T and H proteins only catalyze reactions that form parts of the overall activity of GDC. In the absence of P protein glycine decarboxylation will not occur. This means that the T, H, and L proteins would be redundant in the mesophyll cells of M. arvensis.

In further studies of M. arvensis, it is apparent that the ontogenetic development of GDC subunits is not necessarily coordinated with the development of C_3-C_4-like bundle-sheath tissue (Rylott et al. 1998). The GDC H-protein content of leaves increases from the earliest stages of leaf development, independent of developmental pattern in bundle-sheath. The increase in GDC P-protein, however, is synchronous with the ontogenetic development of mitochondria in the bundle sheath. This occurs when leaf length is between 6 and 12 mm, and there is a bundle sheath-specific, four-fold increase in the number of mitochondria followed by a doubling of their individual profile area. The P-protein is confined to bundle-sheath mitochondria throughout leaf development and its content in individual mitochondria increases prior to the anatomical development of other aspects of the bundle sheath. In leaves and cotyledons that develop in the dark the expression of the P-protein and the organellar development is reduced, but the bundle-sheath cell-specificity is retained.

On the basis of the discovery that GDC activity is largely, if not completely, confined to the bundle-sheath cells of C_3-C_4 intermediate species, Rawsthorne et al. (1988a) proposed a model for photorespiratory metabolism in this group of plants which developed an earlier suggestion by Monson et al. (1984). The current model describing photorespiration in the C_3-C_4 intermediate species M. arvensis is shown in Fig. 3. Carbon dioxide is released in the bundle-sheath cells during photorespiration and the CO$_2$ released by the mitochondria must therefore pass out through the overlying chloroplasts in order to exit the leaf. The close association of mitochondria and the chloroplasts in the bundle-sheath cells is proposed to improve the extent of CO$_2$ recapture relative to that in C_3 species. In a C_3 leaf about 50% of the released CO$_2$ is recaptured, whereas in the leaf of a C_3-C_4 intermediate species this value can be as high as 75% (e.g. Hunt et al., 1987).

IV. Gas-Exchange Patterns as a Result of the Unique Compartmentation of Photorespiratory Metabolism

One of the unique traits of C_3-C_4 intermediates is the

![Fig. 3. A model for photorespiratory metabolism in the leaves of the C_3-C_4 intermediate species Moricandia arvensis (after Rawsthorne et al., 1988a). The relationship between the metabolism of bundle-sheath and mesophyll cells and the cell-specificity of expression of the subunits of glycine decarboxylase is indicated.](image-url)
biphasic response of the CO₂ compensation point (Γ) to atmospheric O₂ concentration (Keck and Ogren, 1976; Quebedeaux and Chollet, 1977; Apel, 1980; Brown and Morgan, 1980; Holaday et al., 1982; Hattersley et al., 1986; Hunt et al., 1987). In the face of increased O₂, Γ of C₃ plants increases linearly, a response that can be explained on the basis of Rubisco kinetics (Laing et al., 1974). In contrast, C₄ plants exhibit little response to changes in O₂ concentration. The biphasic response in C₃-C₄ intermediate plants is evident as a weaker response to low concentrations compared to higher concentrations (Fig. 4). The breakpoint between these responses is quite variable within and among C₃-C₄ species, and in some cases is not readily apparent (Hattersley et al., 1986). The biphasic response of Γ is of critical importance to explaining any mechanism of C₃-C₄ intermediate function (Monson et al., 1984; Peisker and Bauwe, 1984; Hattersley et al., 1986).

Three explanations of the biphasic response have been offered in past studies. Keck and Ogren (1976) proposed the presence of two carboxylases in the same cell, one with a higher affinity towards inorganic carbon than the other (e.g., PEP carboxylase versus RuBP carboxylase). At low O₂ concentrations, it was hypothesized that the high-affinity carboxylase recycled photorespired CO₂, lowering Γ. At higher O₂ concentrations, it was thought that this recycling system would become CO₂-saturated as the rate of photorespiration increased, allowing Γ to increase according to a different linear relationship. Peisker and Bauwe (1984) modified this model to be consistent with data at the time, which suggested the presence of a limited, but complete, C₄ cycle in the most well-studied C₃-C₄ intermediate, Panicum milioides (Rathnam and Chollet, 1979). In the Peisker and Bauwe model, the C₄ cycle effectively recycles photorespired CO₂ at low O₂ concentrations, but becomes constrained by low PEP regeneration rates, at higher O₂ concentrations, resulting in higher CO₂ losses and a different linear response for Γ. This model lost support, however, when it was definitively shown that there was no functional C₄ cycle in Panicum milioides (Edwards et al., 1982).

Using the qualitative model proposed by Rawsthorne et al. (1988a)(Fig. 3), von Caemmerer (1989) developed a quantitative model of CO₂ flux rate in C₃-C₄ intermediate species. The model assumes the glycine shuttle and CO₂ recycling mechanism as discussed above. Using this model, von Caemmerer (1989) showed that the biphasic response of Γ could be explained with a variety of assumptions (Fig. 5). In essence, at low O₂ the recycling of photorespired CO₂ in the bundle-sheath cells is highly efficient and Γ is relatively low. As O₂ concentration increases, this system becomes overwhelmed and more photorespired CO₂ leaks out, increasing Γ. The predicted response is more curvilinear than linearly biphasic, but as pointed out by von Caemmerer (1989) the empirical results that have been collected to date are not of high enough quality to confidently discern details of the shape this response. Using the glycine shuttle model, it can be shown that as the fraction of total leaf Rubisco partitioned to the bundle-sheath cells increases, the extent of the low-O₂ phase becomes greater. This is because there is more Rubisco in the bundle-sheath cells to recycle photorespired CO₂. Likewise, the low O₂ phase can be extended by assuming lower conductances to CO₂ leakage from the bundle-sheath cells (Fig. 5).

Recycling photorespired CO₂ through a glycine shuttle has the potential to increase the light-saturated CO₂ assimilation rate per unit of leaf area (A_{max}).
Because the bundle-sheath tissue of C_3 plants tends to exhibit low photosynthetic activity, increasing the capacity of this tissue for CO₂ assimilation in C₃-C₄ intermediates would increase the potential carboxylation rate per unit leaf area. This assumes that increasing the capacity for CO₂ assimilation of the bundle-sheath tissue occurs with no cost to CO₂ assimilation capacity of the mesophyll. In essence, the C₃-C₄ intermediate state allows for more photosynthetic machinery to be packed into each unit of leaf area. Even without a change in the total carboxylation capacity of the leaf, however, the glycine shuttle can potentially result in increased carboxylation efficiency.

Using the model proposed by von Caemmerer (1989), it can be shown that with the proper balance between glycine decarboxylation rate and Rubisco carboxylation rate in the bundle sheath, CO₂ partial pressures in the bundle sheath can be an order of magnitude higher than in the intercellular air spaces. By allocating a small fraction of the leaf’s Rubisco to the bundle sheath, as opposed to the mesophyll, it operates in an environment that reduces competitive inhibition by the oxygenase reaction and increases the rate of carboxylation (Fig. 6). As is evident in the data of Fig. 6, the advantage to A_max only occurs when a small fraction (e.g., <15%) of the leaf’s Rubisco is allocated to the bundle sheath. At higher fractions, A_max is inhibited because (1) as more Rubisco is sequestered in the bundle sheath there is less oxygenase activity in the mesophyll and thus less glycine shuttled to the bundle sheath, and (2) as the amount of Rubisco in the bundle sheath increases it overwhelms the capacity of the glycine shuttle to provide CO₂, and given limited access to intercellular CO₂, the carboxylation rate of Rubisco decreases (von Caemmerer, 1989).
On the basis of the simulation presented in Fig. 6, one would predict only small improvements (e.g., 10%) in $A_{\text{max}}$ due to the presence of a glycine shuttle. However, it is important to note that this simulation was conducted at 25 °C. At higher temperatures, the ratio of oxygenase to carboxylase activity increases, and there is greater potential for the glycine shuttle to increase $A_{\text{max}}$. At intercellular CO$_2$ partial pressures ($p_i$) typical of C$_3$ and C$_4$ species (e.g., 25 Pa) $A_{\text{max}}$ is predicted to be 23–32% higher in C$_4$ species at leaf temperatures between 35–40 °C, compared to C$_3$ species (Fig. 7). At lower intercellular CO$_2$ partial pressures, this difference increases to reflect a 71–87% improvement in C$_4$ species, compared to C$_3$ species. The interactions among leaf temperature, $p_i$, and mode of carbon assimilation can be further seen in the data presented in Fig. 8. In this case, light-saturated photosynthesis rates for the C$_4$ species are predicted to be higher than those for the C$_3$ species at all values for $p_i$, a difference that increases at 35 °C, relative to 25 °C.

Improvements in $A_{\text{max}}$ through the C$_1$-C$_4$ intermediate mechanism occur without a need for increasing $p_i$ through increased stomatal conductance. As discussed above, internally-generated, photorespired CO$_2$ is re-assimilated. Such a mechanism would be advantageous in that improvements should occur in photosynthetic water-use efficiency (PWUE; CO$_2$ assimilated per unit of H$_2$O transpired). Once again, given that the enhancement of $A_{\text{max}}$ depends upon re-cycling of photorespired CO$_2$, improvements in PWUE should be greatest at warm leaf temperatures (Fig. 9). As an alternative to variation in $p_i$, $A_{\text{max}}$ in C$_3$ plants is influenced by the fraction of leaf nitrogen allocated to Rubisco. Greater allocation to Rubisco would increase the potential rate of carboxylation per unit of leaf area, but unless there are concomitant increases in stomatal conductance, $p_i$ will decrease and the increased $A_{\text{max}}$ will occur at a cost to photosynthetic nitrogen-use efficiency (PNUE; CO$_2$ assimilated per unit of N allocated to Rubisco). As discussed above, it is likely that a fraction of the
Rubisco in C₃-C₄ intermediates operates with greater carboxylation efficiency, and thus greater PNUE, than that for C₃ species. This enhanced carboxylation efficiency is dependent on the re-cycling of photorespired CO₂, and thus any improvements in PNUE should be greatest at warm leaf temperatures. This can be demonstrated using the von Caemmerer (1989) model of C₃-C₄ intermediates (Fig. 9).

At this time it is not entirely clear as to the adaptive advantages, if any, imparted by C₃-C₄ intermediate patterns of CO₂ assimilation. However, as demonstrated by the model simulations described above, it is possible that the re-cycling of photorespired CO₂ provides advantages in terms of $A_{\text{max}}$, PWUE, and PNUE. In gas-exchange studies of different C₃-C₄ intermediate species, advantages of these types have been noted at low $p_i$ values (Brown and Simmons, 1979; Brown, 1980; Hattersley et al., 1986). To date, however, the advantages in terms of enhanced $A_{\text{max}}$ at the $p_i$ values characteristic of normal atmospheric CO₂ conditions (e.g., $p_i = 25$ Pa and $p_o = 36$ Pa), have not been observed for C₃-C₄ species, compared to related C₃ species. The model of von Caemmerer (1989) can be used to predict a 12–25% enhancement in $A_{\text{max}}$ at 25 °C, depending upon specific parameterization values. Measurements have generally revealed no significant differences in $A_{\text{max}}$ between C₃-C₄ and C₃ congeners in this temperature range. At present it is not clear whether the model reflects an unrealistic situation, or whether congeneric differences other than the mode of CO₂ assimilation might offset the predicted differences in $A_{\text{max}}$.

V. The Biochemical Intermediacy of Flaveria C₃-C₄ Intermediates and its Relationship to Reductions in Photorespiration

There is clear evidence that C₃-C₄ intermediates in the genera Alternanthera, Moricandia, Panicum and Parthenium do not have a C₄ cycle which could account for their low rates of photorespiration. It is assumed that the C₃-C₄ intermediate traits associated with these species are due to the glycine shuttle described above. Evidence against the existence of C₄ photosynthesis in C₃-C₄ intermediates of these genera comes from studies of the initial products of $^{14}$CO₂ assimilation by leaves, and of the activities and cellular locations of C₄-cycle enzymes (as discussed in Rawsthorne et al., 1992). Label from $^{14}$CO₂ is not transferred from compounds to Calvin cycle intermediates during photosynthesis. Activities of PEP carboxylase and the C₄ cycle decarboxylases are far lower than in C₄ leaves, and RuBP carboxylase and PEP carboxylase are both present in mesophyll and bundle-sheath cells. Early work on Mollugo verticillata suggested that limited C₄ metabolism might operate in this species (Sayre et al., 1979). However, more recent assessment of this data (Edwards and Ku, 1987) suggests that C₄ photosynthesis is unlikely to account for the C₃-C₄ intermediate gas exchange traits of this species.

C₃-C₄ species in the genus Flaveria exhibit patterns of $^{14}$CO₂ assimilation that reflect considerable C₄-cycle activity (Monson et al., 1986). However, in most of these Flaveria species the presence of the C₄ cycle is ineffective at reducing the level of O₂.
inhibition of $A_{max}$, and on the basis of quantum-yield analyses, there is evidence of futile cycling of CO$_2$ between the C$_3$ and C$_4$ cycles and ineffective CO$_2$-concentrating activity (Fig. 10). Thus the C$_4$ cycle, while being present in these intermediate species, functions inefficiently. At this time, reductions in photorespiration in most C$_3$-C$_4$ Flaveria species cannot be ascribed to reductions in RuBP oxygenase activity through a concentrating mechanism. In addition to the presence of C$_4$-like biochemistry, C$_3$-C$_4$ Flaveria species possess the glycine shuttle described above (Hylton et al., 1988). It is possible that the reduced photorespiration rates of these biochemically-intermediate species are due to the glycine shuttle, and have no relationship to the presence of C$_4$-like biochemistry. Further studies of this issue are warranted.

In some C$_3$-C$_4$ Flaveria species, there is evidence for reduced RuBP oxygenase activity, possibly due to the presence of inefficient, though effective, C$_4$-like biochemistry. In Flaveria ramosissima, 50–60% of the atmospheric CO$_2$ assimilation occurs through the C$_4$ pathway (Rumpho et al., 1984; Monson et al., 1986). Gas-exchange measurements with this species also reveal evidence for reductions in the inhibition of $A_{max}$ and the quantum yield for CO$_2$ uptake by 21 kPa O$_2$ (Rumpho et al., 1984; Monson et al., 1986). However, this species also exhibits differential compartmentation of GDC (Moore et al., 1988). Thus, while a reduction in the O$_2$ inhibition of photosynthesis is likely to be due to the presence of C$_4$-like CO$_2$-concentrating activity, the potential role of a glycine shuttle cannot be excluded. Moreover, the C$_4$-like CO$_2$ concentrating activity may not completely account for the observed low CO$_2$ compensation point. A similar case can be made for the so-called C$_4$-like C$_3$-C$_4$ intermediate Flaveria species (Monson et al., 1987; Moore et al., 1989). In these species 80–90% of the atmospheric CO$_2$ assimilation occurs through the C$_4$ pathway. Additionally, one can observe evidence for significant suppression of RuBP oxygenation in 21 kPa O$_2$. Once again, it is likely that the C$_4$-like CO$_2$-concentrating activity is responsible for a fraction of the lower CO$_2$ compensation points in these species. However, the precise contribution of the C$_4$ cycle relative to the glycine shuttle is not known.

There are anomalous metabolic patterns associated...
with the assimilation of CO$_2$ in C$_3$-C$_4$ *Flaveria* species that remain to be explained. For example, in contrast to C$_3$ and C$_4$ species, significant quantities of $^{14}$CO$_2$ are assimilated into glycine, serine and, rather unusually, fumarate in leaves of C$_3$-C$_3$ *Flaveria* species (Monson et al., 1986). The appearance of $^{14}$C in fumarate is likely to be due to the presence of the large fumarate pool which is present in their leaves (Rawsthorne et al., 1992). The maximum catalytic activities of enzymes involved in C$_4$ photosynthesis (PEP carboxylase, NADP-malate dehydrogenase, NADP-malic enzyme and pyruvate, Pi dikinase (PPDK) are reported to be greater in a number of C$_3$-C$_4$ *Flaveria* species than in C$_3$ species or C$_3$-C$_4$ species from other genera (Ku et al., 1983). However, these activities are only 10–15% of those extracted from *Flaveria* species which have been classified as C$_4$ or C$_4$-like (Ku et al., 1991). In addition, compartmentation between bundle-sheath and mesophyll cells of the enzymes involved in the carboxylation and decarboxylation stages of the C$_4$ cycle is not complete or does not occur in leaves of *F. ramosissima* (C$_3$-C$_4$) or *F. brownii* (C$_4$-like) as judged from protoplast fractionation experiments. The latter species has maximum catalytic activities of PEP carboxylase, PPDK and NADP-ME which approach those in true or other C$_4$-like species which have more limited (5–10 %) direct fixation of CO$_2$ by Rubisco by Moore et al., 1989). There is no marked differential distribution of Rubisco or PEP carboxylase between mesophyll and bundle-sheath in any C$_3$-C$_4$ *Flaveria* species examined to date (Bauwe, 1984; Reed and Chollet, 1985). Enrichment of Rubisco in the bundle-sheath relative to PEP carboxylase and vice versa for the mesophyll has been reported for *F. brownii*. However, without complete separation of the enzymes involved in the carboxylation and decarboxylation phases of the C$_4$ pathway futile cycling of CO$_2$ through C$_4$ acids and hence extra energy consumption may occur.

In terms of gas-exchange, the C$_3$-C$_4$ *Flaveria* species exhibit many of the same advantages reported for species with only a glycine shuttle. In a study of C$_3$-C$_4$ intermediate *Flaveria* species, Monson (1989b) noted improvements in PNUE at low $p_i$, but no improvements in PNUE in two out of the three species studied, when compared to a C$_3$ congener. The one C$_3$-C$_4$ species that exhibited improved PNUE is *F. ramosissima*, a species with reduced O$_2$ inhibition of photosynthesis, and presumably a somewhat effective CO$_2$ concentrating mechanism. In the C$_3$-C$_4$ intermediate *Flaveria floridana*, improvements in $A_{\text{max}}$ at warm leaf temperatures have been observed, even at normal $p_i$ values (Schuster and Monson, 1990; Monson and Jaeger, 1991). It is not clear whether these improvements are due to the glycine shuttle system, or a C$_4$-like CO$_2$-concentrating mechanism.

VI. Intercellular Metabolite Movement in C$_3$-C$_4$ Leaves

The partitioning of photorespiratory metabolism between bundle-sheath and mesophyll cells in the leaves of C$_3$-C$_4$ species has a number of implications for the movement of metabolites between the two cell types. The first relates to how the metabolites are transported between the cells. Studies of leaf anatomy in C$_3$-C$_4$ intermediate species (Brown and Hattersley, 1989) reveal that glycine produced during photorespiratory metabolism in mesophyll cells may have to move over several cell distances in order to be decarboxylated in bundle-sheath cells. How this glycine shuttle is facilitated is not known but exchange between bundle-sheath and mesophyll is likely to be via plasmodesmatal interconnections as described above. Rawsthorne and Hylton (1991) have speculated that glycine may move down a concentration gradient from the mesophyll to the bundle-sheath based upon measurements of glycine pool size during light/dark transitions and on the localization of GDC to the bundle-sheath cells. The change in glycine content of C$_3$-C$_4$ leaves during the immediate post-illumination period and of the labeling of glycine during pulse-chase experiments with $^{14}$CO$_2$, indicate that the glycine pool is a result of photorespiration and is much larger in C$_3$-C$_4$ than in C$_3$ leaves (Holaday and Chollet, 1983; Monson et al., 1986; Rawsthorne and Hylton, 1991).

A second implication of the C$_3$-C$_4$ glycine shuttle (Fig. 3) is that leaf nitrogen metabolism must be different from that in C$_3$ species. The GDC reaction produces ammonia which would need to be reassimilated. In a C$_3$ leaf, this assimilation occurs in all photorespiring cells. In a C$_3$-C$_4$ leaf, however, the same rate of reassimilation occurs in a much smaller proportion of the leaf cells (i.e. in the bundle-sheath). This would require that nitrogen be returned to the mesophyll cells. To date there is no definitive evidence that the activity of enzymes required for the reassimilation of ammonia are elevated in bundle-
sheath, as compared to mesophyll, cells in the leaves of C₃-C₄ species. However, where reported, the activities would be high enough to account for the ammonia flux in vivo which can be estimated from the rate of photorespiratory CO₂ release (Rawsthorne et al., 1988b).

The production of serine by GDC/SHMT, and the requirement to reassimilate ammonia, in the bundle-sheath cells are clear consequences of the metabolic scheme proposed in Fig. 3. However, it is unclear in what form the carbon and nitrogen that is lost from the mesophyll cells, in the form of glycine efflux, is returned. Rawsthorne et al. (1992) have argued that return of carbon to the mesophyll is essential if the plant is to maintain flux through the photosynthetic carbon reduction cycle. It is possible that serine produced in the bundle-sheath could diffuse back to the mesophyll and so return one of the two nitrogens and three of the four carbon atoms derived from the original two molecules of glycine. How or whether the nitrogen balance between mesophyll and bundle-sheath is maintained is not known.

VII. Carbon Isotope Discrimination Patterns in C₃-C₄ Intermediates

The recycling of photorespired CO₂ should cause a shift in the carbon isotope composition of assimilated carbon. This is because Rubisco in the bundle sheath cells of C₃-C₄ intermediates has the opportunity to discriminate against ¹³CO₂ that has been released through decarboxylation of glycine that already carries a 'light' δ¹³C value relative to the atmosphere. Thus, the total discrimination against ¹³C should be slightly higher in C₃-C₄ intermediate plants, compared to C₃ plants. The level of isotopic discrimination should increase at low p₄ values, since the fraction of recycled CO₂ to total assimilated CO₂ will increase (von Caemmerer, 1989). These predictions have been tested using on-line, isotopic fractionation measurements. When measured with leaves of Panicum milioides, a C₃-C₄ intermediate species that possesses the glycine shuttle, short-term discrimination against ¹³C was not significantly different from C₃ species at normal, ambient CO₂, but was substantially higher at low CO₂ (Fig. 11). It is not clear why the presence of the glycine shuttle does not affect the discrimination signature at normal, ambient CO₂. However, it is consistent with the fact that modeled influences of the glycine shuttle, whether they relate to predictions of Aₚₚₚₚ or discrimination against ¹³C, are difficult to observe at normal, ambient CO₂.

In those C₃-C₄ intermediate species that possess C₄-like biochemistry, discrimination against ¹³C is influenced, not only by the presence of the glycine shuttle, but also by the degree to which the C₄ and C₃ cycles are integrated (Monson et al., 1988). An apparent enigma occurs in that C₃-C₄ Flaveria species with considerable capacity to assimilate atmospheric CO₂ through the C₄ pathway, exhibit C₃-like carbon isotope ratios (expressed as δ¹³C values). In an early analysis, Peisker (1985) explained this pattern by invoking a PEP-regeneration constraint to the activity of PEP carboxylase, and minimizing assimilation of
atmospheric CO$_2$ through the C$_4$ pathway. With this model, C$_4$-like $\delta^{13}$C values are the result of C$_4$ photosynthetic activity that is too low to significantly affect the carbon isotope signature.

In an alternative approach, Monson et al. (1988) allowed for significant rates of CO$_2$ assimilation through the C$_4$ pathway, but invoked a low degree of coordination between the C$_4$ and C$_3$ cycles. Thus, the C$_4$ system is 'leaky'. In other words, CO$_2$ that is assimilated and then released through decarboxylation by the C$_4$ pathway has a limited potential to accumulate at the site of RuBP carboxylation. High leakage of CO$_2$ from the site of RuBP carboxylation provides RuBP carboxylase the opportunity for discrimination against $^{13}$C, and shifts the predicted $\delta^{13}$C value toward the C$_4$-like extreme (Fig. 12). Using a model based on the calculation of CO$_2$ leakage rates from measured quantum yields, Monson et al. (1988) predicted only small shifts in the $\delta^{13}$C value of C$_3$-C$_4$ intermediate Flaveria species from the C$_3$extreme, even for those species that assimilated up to 50% of their carbon through the C$_4$ pathway. In Flaveria, effective integration of the C$_3$ and C$_4$ cycles only occurs in species that assimilate more than 60% of their carbon through the C$_4$ pathway (Fig. 13). This suggests that there is an upper limit beyond which the evolution of increased activity of an inefficient, poorly integrated C$_4$ cycle becomes too costly. In Flaveria, this limit appears to be the point at which more than 50% of the assimilated carbon occurs through the C$_4$ pathway. Above this limit, selection tends to improve integration between the C$_4$ and C$_3$ pathways at the same time that the fractional magnitude of C$_4$ assimilation increases.

VIII. C$_3$-C$_4$ Intermediates and the Evolution of C$_4$ Photosynthesis

There has been much speculation as to the significance of C$_3$-C$_4$ intermediates in the evolutionary transition from the C$_3$ to C$_4$ state (e.g. Monson and Moore, 1989; Monson 1996, 1999). As discussed above, CO$_2$ assimilation through the C$_4$-C$_4$ pathway can give rise to improved photosynthetic performance at warm temperatures when comparisons are made with C$_4$ plants (Schuster and Monson, 1990). Improved gas exchange characteristics at such temperatures could translate into improved fitness and so provide the basis for the evolution of more C$_4$-like traits.
In recognizing this possibility one cannot be certain of the driving force behind such an evolutionary event, nor can one be certain of the steps in which such evolution occurred. The evolution of $C_3-C_4$ intermediate traits could have improved water-use-efficiency, reduced photorespiratory $CO_2$ loss, or both, and thereby contributed to adaptive advantages in comparison to $C_3$ progenitors. This would have been particularly true if, as predicted in paleoclimate models, atmospheric concentrations have decreased since the Miocene, thereby increasing photorespiration rates relative to photosynthesis rates (Ehleringer et al., 1991).

Based upon our knowledge of metabolism and gas exchange in present day $C_3$, $C_3-C_4$, and $C_4$ species across and within genera it is reasonable to speculate upon how $C_4$ photosynthesis might have evolved from the older $C_3$ mechanism. By drawing upon the spectrum of $C_3-C_4$ phenotypes which exist, a sequence of discrete steps is apparent, each representing a progressive change towards $C_4$ photosynthesis (Fig. 14). Whether such steps actually occurred we do not know, but the fact that they are drawn from across the higher plant phylogenetic tree suggests that the polyphyletic evolution of $C_4$ photosynthesis may have had common mechanistic origins.

The similar anatomical development of bundle-sheath cells and the consistent absence of GDC from mesophyll cells in all $C_3-C_4$ species studied to date indicates that the differential distribution of GDC could have been a primary event in the evolution of $C_4$ photosynthesis. In Panicum laxum the partial loss of GDC from the mesophyll (Hylton et al., 1988) and partial development of the bundle-sheath cells (Brown et al., 1983) are suggestive of this species representing an early step in this process. This step could have been followed by total loss of at least one of the GDC proteins from the mesophyll, as in M. arvensis followed by more complete loss of GDC proteins as seen in other $C_3$-$C_4$ species. Consistent with this hypothesis, GDC in $C_4$ species is, as in $C_3$-$C_4$ species, confined to bundle-sheath cells (Ohnishi and Kanai, 1983; Hylton et al., 1988).

How the evolution of true $C_4$ metabolism, as may be represented in the genus Flaveria (Fig. 13), occurred upon this base of partitioning of photorespiratory metabolism is unclear. The common requirements of the $C_4$ and $C_3$-$C_4$ intermediate pathways for intercellular metabolite exchange represent a possible link between the two mechanisms. Perhaps $C_4$ metabolism evolved initially as a consequence of the partitioning of photorespiratory metabolism and the consequences for C and N exchange between the bundle-sheath and mesophyll. Recruitment of existing ‘$C_3$ genes’ and changes in their expression in $C_4$ species is certainly evident (Ku et al., 1996; Monson, 1999). The studies of genes encoding $C_4$ enzymes have revealed that in some instances the expression of single orthologous gene present in $C_3$, $C_3$-$C_4$, and $C_4$ species has been increased and targeted to a specific cell type in the $C_4$ species. In other cases the ancestral ‘$C_3$ gene’ has
been replicated and the expression of the replicated gene has then been modified to provide a C₄-type expression pattern (see Ku et al., 1996 for details). Recent studies of the *Flaveria* species have, however, provided the first convincing evidence that plants do indeed represent intermediates in the evolution between C₃ and C₄ photosynthesis. One of the GDC subunits, the moderately conserved H-protein, has now been used as molecular marker to examine phylogenetic relationships within the genus (Kopriva et al., 1996). A phylogenetic tree of these sequences comprises three main clusters: (i) the C₃ species *F. pringlei* and *F. cronquistii*, (ii) all C₃-C₄ species including the C₄-like *F. brownii*, and (iii) the advanced C₄ species *F. australasica*, *F. trinervia*, *F. bidentis*, and *F. palmeri* (Kopriva et al., 1996). The ancient position of C₃ photosynthesis in the genus is clearly reflected, as well as a twofold and independent evolution of C₄ photosynthesis in different lineages. Perhaps more importantly these data provide direct evidence for the phylogenetically intermediate position of C₃-C₄ intermediates.

**IX. Concluding Statement**

Knowledge about the metabolic details of C₃-C₄ intermediates has crystallized as a result of research over the past decade and a half. It is now clear that many of the C₃-C₄ intermediate traits can be explained on the basis of the glycine shuttle, originally elucidated by Rawsthorne et al. (1988a). This
mechanism appears to be ubiquitous among C\textsubscript{3}-C\textsubscript{4} intermediate species, at least those of terrestrial origin. (Some aquatic plants have been shown to exhibit C\textsubscript{3}-C\textsubscript{4} intermediate traits, but the underlying mechanisms appear to differ with respect to the glycine shuttle described above; e.g., see Bowes this volume.) There are still significant uncertainties that must be resolved before a complete understanding of the glycine-shuttle mechanism is understood. For example, it is still unclear as to whether the CO\textsubscript{2} concentration of bundle-sheath cells in C\textsubscript{3}-C\textsubscript{4} plants is increased during light-dependent CO\textsubscript{2} assimilation. It is also unclear as to why the predicted influences of a glycine shuttle on leaf-level CO\textsubscript{2} assimilation rates, and water- and nitrogen-use efficiency, are not observed when actual measurements are made at normal, ambient CO\textsubscript{2} concentrations.

The functional significance of the C\textsubscript{4}-like biochemistry found in C\textsubscript{3}-C\textsubscript{4} Flaveria intermediates is also uncertain. It is possible that traits such as a low CO\textsubscript{2} compensation point and the biphasic response of the CO\textsubscript{2} compensation point to changes in ambient O\textsubscript{2} concentration are due to the glycine shuttle in the Flaveria intermediates. Other traits such as reduced O\textsubscript{2} inhibition of photosynthesis cannot be readily explained by the glycine shuttle and are likely due to the limited CO\textsubscript{2}-concentrating capacity of the C\textsubscript{4}-like biochemistry (Moore et al., 1987). However, the precise nature of any CO\textsubscript{2}-concentrating activity in C\textsubscript{3}-C\textsubscript{4} Flaveria species is still equivocal. Likewise, it is unclear as to how intracellular CO\textsubscript{2} concentrations can be increased given the lack of C\textsubscript{4}-like partitioning of the C\textsubscript{4} and C\textsubscript{1} cycles between mesophyll and bundle-sheath cells.

Finally, many of the uncertainties involved with the evolutionary status of C\textsubscript{3}-C\textsubscript{4} intermediate plants can now be resolved using phylogenetic reconstruction based on molecular sequence data from specific proteins (e.g., Kopriva et al., 1996). This type of insight will be of critical importance as we attempt to (1) reconstruct specific metabolic paths to C\textsubscript{4} photosynthesis, (2) place the evolution of C\textsubscript{4} photosynthesis into the context of past ecological and biogeochemical events (e.g., global-level dynamics in atmospheric concentrations and Neogene expansion of global grasslands), and (3) attempt to identify the significant selective forces that have driven plants to this alternative mode of carbon assimilation.

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Russell K. Monson and Stephen Rawsthorne
Summary

Crassulacean acid metabolism (CAM) represents a notable adaptation to photosynthetic CO₂ fixation that affords plants a competitive advantage in CO₂- or water-limiting environments. The physiological and biochemical characteristics of CAM are well understood, as are the ecophysiological implications of the pathway. As our knowledge expands, it has become increasingly evident that the CAM adaptation exhibits an enormous degree of metabolic plasticity, especially in the extent to which CAM is influenced by environmental...
I. Introduction

Two distinct metabolic pathways for sequentially assimilating and concentrating atmospheric CO\textsubscript{2} exist in approximately 10% of higher plants—C\textsubscript{4} photosynthesis and CAM. The driving forces responsible for the evolution of these photosynthetic adaptations may include limited CO\textsubscript{2} availability due to ancient decreases in global atmospheric CO\textsubscript{2} concentrations, high light and temperature conditions, lack of water, or a combination of these environmental stress factors. Under heat and water stress, CO\textsubscript{2} often becomes limiting when stomata close, which leads to increased photorespiration, a consequence of the incomplete discrimination of CO\textsubscript{2} over O\textsubscript{2} by Rubisco. C\textsubscript{4} and CAM plants can suppress photorespiration by utilizing a ‘CO\textsubscript{2} pump’ involving PEPC that elevates intracellular CO\textsubscript{2} concentrations in the vicinity of Rubisco. In C\textsubscript{4} plants, this concentration occurs through the spatial separation of PEPC and Rubisco between two cell types (Furback and Taylor, 1995; Ku et al., 1996). C\textsubscript{4} photosynthesis requires a developmental program by which cell-specific differentiation patterns form two distinct photosynthetic cell types, mesophyll and bundle sheath. The genetic modifications required for the generation of these cell types in the C\textsubscript{4} leaf are considered extensive (Ku et al., 1996; Chapter 20, Dengler and Taylor), or, at least, more substantial than the changes stipulated for the establishment of CAM carried out in mesophyll cells alone. This notion is supported by phylogenetic studies which suggest that CAM evolved earlier and is more widespread than C\textsubscript{4} photosynthesis (Ehleringer and Monson, 1993; Raven and Spicer, 1996; Smith and Winter, 1996). CAM plants perform both carboxylation reactions within mesophyll cells, but the reactions, which have been described as a sequence of four individual phases, are separated temporally (Osmond, 1978; Winter and Smith, 1996; Cushman and Bohnert, 1997).

Emergence of the CAM pattern also demanded numerous genetic modifications, but evolution favored alterations in diurnal or circadian regulation, modifications of intracellular partitioning of photosynthetic products, and changes in metabolic signaling for the regulation of the two competing carboxylation reactions within a single cell. Additionally, expression of the pathway can vary extensively during development, in response to environmental conditions, and among phylogenetically related CAM-performing species. The anatomical, phylogenetic, evolutionary, ecological, physiological, and biochemical aspects of CAM have
II. Permutations and Metabolic Plasticity of CAM

A striking feature of CAM is the extreme physiological and metabolic plasticity of the pathway and the degree to which pathway expression can be influenced by developmental or environmental factors. The extent of malate cycling, carbohydrate storage, and the contribution of nocturnal CO₂ fixation to net carbon gain can vary substantially. Thus, different CAM species can range from nearly C₄ to obligate CAM, including facultative CAM species that swing into and out of CAM depending on changes in environmental conditions or developmental status (Lüttgé, 1993; Edwards et al., 1996; Smirnoff, 1996; Ting et al., 1996). In some obligate CAM species, net CO₂ uptake occurs almost exclusively at night even under well watered conditions. Other CAM plants appear nearly-C₃ and usually exhibit net CO₂ uptake only during the day. This mode of CAM, referred to as CAM cycling, is characterized by the appearance of diurnal fluctuations in C₄ acid levels in the absence of daytime stomatal closure and little or no net nocturnal carbon assimilation (Ting, 1985). By refixing respiratory CO₂ at night, plants that perform CAM-cycling are thought to be poised to enter full CAM rapidly when called for by prevailing environmental conditions. Another variation, called CAM-idling, occurs under extreme drought stress conditions when stomata remain closed day and night, yet diurnal fluctuations in organic acids continue as a result of refixation of respiratory CO₂ (Ting, 1985, 1987; Patel and Ting, 1987; Bastide et al., 1993). CAM-idling may help preserve the activities of photosynthetic enzymes until favorable growth conditions return. Between the two extremes of CAM-cycling and CAM-idling are found various patterns of gas exchange and C₄ acid fluctuation that fall within a continuum of the four phases of CAM (Osmond, 1978; Leegood and Osmond, 1990; Smith and Bryce, 1992) as dictated by a complex interplay of environmental and developmental parameters (Borland and Griffiths, 1996).

III. Control of CAM Induction

Facultative CAM plants shift from C₃ to CAM or different modes of CAM (e.g., CAM-cycling or CAM-idling) in response to developmental cues or environmental signals, such as photoperiod length, high salinity, or water deficit, and plant growth regulators. The ability to shift into CAM is present in a wide variety of species from diverse families. The inducible pathway is predominantly found among the Aizoaceae, Clusiaceae, Crassulaceae, Vitaceae, Piperaceae, and Portulacaceae (Winter and Smith, 1996). Inducible CAM-like behavior has also been reported in the C₃ genus Portulaca (Mazen, 1996; Kraybill and Martin, 1996). CAM has also been recognized in several genera of aquatic plants including Isoetes, Crassula, Littorella, and Sagittaria (Keeley, 1996). In aquatic environments, CAM enhances inorganic carbon acquisition under conditions of limited CO₂ availability (Raven and Spicer, 1996). In Littorella uniflora (shoreweed), which grows partially inundated along the margins of lakes, CAM is induced by rehydration of the plants which perform C₄ photosynthesis when not immersed in water (Robe and Griffiths, 1992). In agreement with these observations, the ratio of PEPC:Rubisco activity was found to be consistently higher under submerged rather than emergent conditions (Keeley, 1996). This behavior is in contrast to the usual induction of CAM by water-limiting conditions. The exact nature of the signals that regulate CAM activity in aquatic environments remains an open question and requires further attention.

In this section, we will focus on the role that development, environmental stress, and plant growth regulators have on CAM induction in three different model CAM genera: Peperomia ssp., Kalanchoë,
and *Mesembryanthemum*. Developmental aspects of CAM induction have been in the foreground of studies using *Peperomia* ssp. (Ting et al., 1996), whereas photoperiodic induction and circadian rhythms of CAM have been analyzed most comprehensively in *Kalanchoë* (Brulfert et al., 1985, 1988; Wilkins, 1992). Since the initial discovery that high salinity can serve as a reliable trigger to switch *M. crystallinum* from C₃ photosynthesis into CAM (Winter and von Willert, 1972), this plant has become an important model for studying how CAM is induced (Cushman and Bohnert, 1997) and other aspects of ecological adaptation to environmental stress (Adams et al., 1998).

**A. Development**

Developmental status plays an important role in modulating the timing and extent to which CAM is expressed (Kluge and Ting, 1978; Winter, 1985). An age-dependent shift from C₃ photosynthesis to CAM has been documented for many *Peperomia* species, which provide an excellent model for the study of CAM ontogeny since, during normal development, leaves show a progressive expression of CAM through the sequence: C₃ → CAM cycling → CAM (Sipes and Ting, 1985; Holthe et al., 1987, 1992; Ting et al., 1996). In *Peperomia scandens* and *P. camptotricha*, leaves are arranged in successive false whorls from young leaves lacking CAM attributes to mature leaves with CAM (Ting et al., 1996). As leaves age, diurnal fluctuations of acidity increased in parallel with PEPC activity, PEPC protein and mRNA accumulation (Ting et al., 1993, 1996). Increased CAM correlated with increased leaf thickness attributable mainly to a thickening and expansion of the spongy mesophyll and multiple epidermis. Increased air spaces within these tissues evidently increase the potential for gas exchange, thus facilitating CO₂ fixation in older leaves (Nobel, 1988). In contrast, palisade mesophyll cells, the site of predominantly C₃ activity, showed no significant changes in size. In older leaves, water stress can stimulate PEPC mRNA accumulation and activity. Upon rewatering, however, PEPC activities return to amounts typical of the constitutive CAM state prior to the imposition of water stress suggesting that the PEPC response to water stress is fully reversible (Ting et al., 1996). Studies exploring the relationship between leaf aging, photoperiod and the induction of CAM in *K. blossfeldiana* and *K. velutina* have also demonstrated that leaf aging promoted CAM under both short- and long-day conditions (Queiroz and Brulfert, 1982; Brulfert et al., 1982b).

In *M. crystallinum*, immature leaves or young plants are less able to induce CAM and CAM-specific genes such as *PpC1* or *Ppdk1* (see Table 1) following environmental stress suggesting that a critical developmental threshold dictates the onset or inducibility of CAM or CAM-specific genes (von Willert et al., 1976a; Ostrem et al., 1987; Cushman et al., 1990; Cheng and Edwards, 1991; Herppich et al., 1992; Bohnert et al., 1994; Füllthaler et al., 1995). However, the timing of CAM induction or CAM-specific gene expression can vary considerably depending on photoperiod length, temperature, nutrient supply, or volume of the rooting medium (Cheng and Edwards, 1991; Schmitt and Piepenbrock 1992a; Piepenbrock et al., 1994) suggesting that environmental factors rather than development dictate CAM induction. Reductions in cell and leaf turgor pressure and water content occur even in well-watered plants with progressing age (Heun et al., 1981; Rygol et al., 1986; Winter and Gademann, 1991) indicating that incremental development of low levels of CAM activity in such plants seems to arise from water stress rather than from a developmental program (Schmitt and Piepenbrock 1992a; Piepenbrock et al., 1994). This is in agreement with earlier observations by Winter (1973b) who documented conditions which maintained high leaf water contents (moderate light intensity and high relative humidity) and effectively postponed the onset of CAM. More recently, split roots experiments have demonstrated roots can perceive water stress and convey this information to leaves triggering a switch from C₃ to CAM photosynthesis without detectable reductions in leaf turgor (Eastmond and Ross, 1997). As in *Peperomia*, stress-mediated induction of PEPC activity, protein or mRNA is reversed in *M. crystallinum* plants or excised leaves following removal of stress (von Willert et al., 1976b; Vernon et al., 1988; Schmitt, 1990; Piepenbrock and Schmitt, 1991; Schmitt and Piepenbrock, 1992b). Whatever their relative contribution to CAM, it is obvious that, at least in certain CAM plants, a combination of developmental and environmental factors influences CAM induction.

**B. Environmental Factors**

Many different environmental factors can influence the extent of nocturnal CO₂ fixation in CAM plants.
### Chapter 23  Crassulacean Acid Metabolism Induction

<table>
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<td>T. Taybi and J.C. Cushman,</td>
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<td><em>M. crystallinum</em></td>
<td>TP</td>
<td>NaCl</td>
<td>Löw et al., 1996</td>
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<td>NaCl</td>
<td>Holtum and Winter, 1982</td>
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<td>–</td>
<td>NaCl</td>
<td>Holtum and Winter, 1982</td>
</tr>
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</table>

1 Citations provided where accession numbers unavailable. Dashes (–) indicate gene is not induced. Question marks (?) indicate information not available. CYT, cytoplasm; CP, chloroplast; MT, mitochondria; PER, peroxisome; TP, tonoplast; NC, nucleus; VAC, vacuole.
Some acute factors, such as drought, salinity and high temperature stress result in rapid, reversible CAM induction, whereas factors such as photoperiod initiate a slow, irreversible induction sometimes coordinated with developmental events such as flowering. Environmental stress induction of CAM occurs in a wide variety of facultative CAM or C₃/C₄ CAM intermediate genera including Mesembryanthemum (Edwards et al., 1996; Cushman and Bohnert, 1996; Schmitt et al., 1996), Clusia (Lüttge, 1996; Zott and Winter, 1996), Kalanchoë (Brulfert et al., 1993; Taybi et al., 1995; Kluge and Brulfert, 1996), Calandrinia (Winter et al., 1981), Cissus (De Santo and Bartoli, 1996), Sedum (Smirnoff, 1996), Peperomia (Ting et al., 1996), and Plectranthus (Herppich et al., 1998).

K. blossfeldiana has been the model for investigating the influence of light intensity, light quality, and duration on the regulation of CAM induction (Brulfert et al., 1988). This species was the first identified as an inducible CAM plant resulting from changes in photoperiod length (Gregory et al., 1954; Queiroz, 1968). Since this discovery, Kalanchoë species have been used to study the effects of light on the diurnal or circadian rhythmicity of CAM induction (Brulfert et al., 1982a,b; Brulfert and Queiroz, 1982). When Kalanchoë plants grown under long-day photoperiods are transferred to short day photoperiods, CAM is induced within seven days, but full expression of the pathway may take 40–60 short days (Brulfert et al., 1975, 1982b). Control by short daylength was also observed in green callus tissue cultures from K. blossfeldiana, however, a lag time of 30–40 days preceded the onset of CAM (Brulfert et al., 1987; Kluge et al., 1987). Short-day induction can be effectively inhibited by interrupting the dark period by exposure to red light (Gregory et al., 1954; Mricha et al., 1990) a process controlled by phytochrome in intact plants (Brulfert et al., 1975, 1985) and green callus tissue of K. blossfeldiana (Mricha et al., 1990). The coupling of photoperiod to CAM induction may provide a means for the plant to measure the advance of the season and thus may be an effective way of establishing the metabolic machinery for CAM prior to the onset of seasonal environmental conditions such as drought (Brulfert et al., 1982b; Queiroz and Brulfert, 1982). In contrast to the rapid induction by salinity or drought stress, the slow photoperiodic induction of CAM in K. blossfeldiana appears to be irreversible (Queiroz and Brulfert, 1982). This observation suggests that the mechanisms by which photoperiodism controls CAM induction may differ fundamentally from those mediating salinity or drought stress induction.

M. crystallinum, the best-studied inducible CAM model, exhibits rapid CAM induction in response to high salinity (Winter and von Willert, 1972; Winter 1973a) as well as other conditions such as drought, high light combined with low humidity, exposure of roots to low temperature, or root anoxia (Winter 1973b, 1985). The induction is associated with large increases in the extractable activity of enzymes associated with malate formation and decarboxylation, glycolysis and gluconeogenesis, and starch synthesis and degradation (Holtum and Winter, 1982, Winter et al., 1983; Paul et al., 1993; see also Table 1). Notably, the activities of some C-6 compound processing enzymes (Holtum and Winter, 1982) or photorespiratory enzymes (Whitehouse et al., 1991) are not induced.

In M. crystallinum, long photoperiods, high light intensity, and phytochrome are associated with accelerating the life cycle of the plant, stimulating malate anion accumulation, and inducing the activity of CAM enzymes such as NADP-malic enzyme and PEPC (Cheng and Edwards, 1991). Although plants exposed to constant illumination accumulated high amounts of malate and key CAM enzymes, they did not exhibit diurnal fluctuations in malate or titratable acidity suggesting that CAM requires a dark period of sufficient length to effect the regulation of enzymes. High light intensity and quality can act synergistically with NaCl or ABA to enhance the expression of the CAM-specific form of PEPC in M. crystallinum (McElwain et al., 1992). Expression is further improved by incandescent light (McElwain et al., 1992) or a low ratio of red:far red light suggesting that phytochrome mediates the enhancement (Cockburn et al., 1996). Plants grown under low red:far red light ratios flowered earlier, exhibited longer internode lengths leading to a closed canopy, and accumulated greater amounts of the osmoprotectant pinitol than plants grown under high red:far red ratios. These responses, along with CAM induction, are thought to be components of a stress response syndrome associated with adaptation to arid conditions (Cockburn et al., 1996).

C. Plant Growth Regulators

Many stresses can lead to pronounced changes in the absolute amount of endogenous plant growth
ABA may be mediated directly through ABA-accumulation in leaves of well-watered plants, and dehydration. It also inhibits PEPC transcript enzyme activity, and CAM induction by salt or cytokinin appears to suppress PEPC transcript, by ABA is influenced by light intensity (McElwain et al., 1992b). Furthermore, the efficacy of CAM induction biosynthesis has been suppressed (Thomas et al., 1992b; Taybi et al., 1995). Endogenous pools of ABA increase during short-day triggering of CAM induction in K. blossfeldiana (Brulfert et al., 1982a; Taybi et al., 1995). Increases in ABA accumulation precede CAM gene activation, whether brought about by drought stress or short-day regimes (Taybi et al., 1995). The concept of assigning a central role to ABA has been reinforced by the observation that exogenous application of ABA can induce CAM (Ting, 1985; Taybi et al., 1995), elevate activities (Chu et al., 1990; Dai et al., 1994; Taybi et al., 1995) and mRNA amounts of CAM enzymes including PEPC (Cushman et al., 1993; Taybi et al., 1995), enolase (Forsthoefel et al., 1995a), PGM (Forsthoefel et al., 1995a), and V-ATPase subunit c (Tsiantis et al., 1996). An analog of ABA and a potent antitranspirant, farnesol, can also trigger CAM induction (Dai et al., 1994). ABA distribution within tissues as well as the absolute content of ABA or ABA conjugates may also be important for CAM induction (Dai et al., 1994). Despite these observations, CAM induction by NaCl stress can also occur in leaves in which ABA biosynthesis has been suppressed (Thomas et al., 1992b). Furthermore, the efficacy of CAM induction by ABA is influenced by light intensity (McElwain et al., 1992). Modulation of CAM gene expression by ABA may be mediated directly through ABA-responsive elements (ABREs), present in the 5’ flanking region of Ppc1, GapC1, and Ppdkl genes (Cushman and Bohnert, 1992; Schmitt et al., 1996).

Cytokinins have been documented to either suppress or mimic stress-induced CAM induction depending on the organ to which the growth regulator is applied. When applied to shoots or detached leaves, cytokinin appears to suppress PEPC transcript, enzyme activity, and CAM induction by salt or dehydration. It also inhibits PEPC transcript accumulation in leaves of well-watered plants, and accelerates the net decrease in PEPC transcripts in excised leaves during dehydration recovery (Schmitt and Piepenbrock, 1992b; Dai et al., 1994; Schmitt et al., 1996). In detached leaves, the suppression of PEPC transcripts or activity is rapid (within 8 h) and occurs in both well-watered and stressed leaves in a dose-responsive manner (Schmitt and Piepenbrock, 1992b; Dai et al., 1994). Cytokinin accumulation is also negatively correlated with PEPC transcript accumulation suggesting that leaf cytokinins act as negative effectors of CAM expression (Peters et al., 1997). It has also been postulated that water stress limits the supply of cytokinin from the roots via the transpiration stream resulting in derepression of PEPC expression (Schmitt and Piepenbrock, 1992b). In contrast, when applied to the rooting medium of soil or hydroponically grown M. crystallinum, cytokinin can mimic environmental stress by increasing the accumulation of PEPC transcripts, protein, and enzyme activity (Thomas et al., 1992b; Dai et al., 1994; Peters et al., 1997). Enolase and PGM transcripts have been shown to be similarly affected (Forsthoefel et al., 1995a, b). Unlike ABA, however, cytokinin accumulation in M. crystallinum leaves declines during salinity stress (Peters et al., 1997). Furthermore, induction occurs slowly over a period of one to seven days and is associated with other salt-induced physiological reactions such as accumulation of proline, polyols, and osmotic (Thomas et al., 1992b; Thomas and Bohnert, 1993; Peters et al., 1997). While cytokinin is known to directly modulate many changes in stress-related gene expression (Harding and Smigocki, 1994; Thomas et al., 1995), its effect may be secondary in nature, possibly resulting from water deficits caused by inhibition of root growth (Stenlid, 1982; Su and Howell, 1992). The opposing effects of the same growth regulator on CAM induction may also depend on the location of cytokinin application and the time of analysis following treatment (Schmitt et al., 1996; Peters et al., 1997). For example, application of cytokinin to leaves suppresses PEPC transcript accumulation stimulated by its simultaneous application to roots (Peters et al., 1997).

The plant growth regulator MJA is an important signal molecule which influences gene expression in a range of processes such as wounding or pathogen attack, osmotic or dehydration stress, and leaf senescence (Sembdner and Parthier, 1993; Reinothe et al., 1994). MJA represses the expression of genes involved in photosynthetic carbon fixation. It has
also been implicated in modulating CAM induction by down-regulating PEPC expression and CAM. Exogenous application of MJA to leaves of *M. crystallinum* for several days inhibited the accumulation of PEPC transcripts, enzyme activity and CAM induction in salt stressed plants. MJA accelerated the decline in PEPC transcript amounts in excised leaves undergoing stress recovery (Schmitt et al., 1996) and inhibited PEPC accumulation in well-watered plants (Dai et al., 1994). When applied together, MJA and 6-BAP have an additive effect on the attenuation of PEPC and CAM induction. Consistent with a role in leaf senescence, treatment of *M. crystallinum* roots with MJA resulted in a strong and rapid induction of sulphydryl endopeptidase expression in leaves (Forsthoefel et al., 1998). This protease may function to recycle amino acids for de novo synthesis of enzymes associated with stress adaptation. MJA also causes global changes in translational activity in response to environmental stress (Reinbothe et al., 1993a, 1994). Such changes are thought to be mediated, at least in part, through post-transcriptional modification of transcripts (Reinbothe et al., 1993a,b) and by stress-induced increases in ribosome inactivating proteins (RIPs) implicated in programmed senescence and stress adaptation (Stipe et al., 1996; Rippmann et al., 1997). The interplay of development, environmental stress, and growth regulators involved in the signal perception and transduction pathways that results in CAM induction is considered in more detail in Section VI.

### IV. Genes, Transcripts, and Proteins

The enzymatic machinery required for CAM is present in all higher plants. In CAM plants, however, the key enzymes responsible for nocturnal CO$_2$ fixation, malate metabolism, glycolysis, and gluconeogenesis are expressed more abundantly than in C$_3$ plants. For example, during the shift from C$_3$ to CAM in *M. crystallinum* increased enzyme activities for less than 15 enzymes have been documented (Holtum and Winter, 1982; Winter et al., 1982). However, it has been estimated that the expression of several hundred genes is altered during this transition with about 100 genes being up-regulated (Meyer et al., 1990). As the discovery of genes from *M. crystallinum* and other CAM plants continues (Table 1), more than 20 genes have been characterized whose expression is enhanced following environmental stress conditions known to induce CAM. In addition, many of these genes and enzymes are tightly regulated on a diurnal or circadian basis. The characterization of CAM-specific genes, gene families, and their expression patterns in facultative CAM plants has begun to shed light on the molecular mechanisms underlying the evolution and expression of genes responsible for CAM induction.

Two classes of CAM-related genes are beginning to emerge from molecular genetic analyses of gene structure and expression patterns. One class of genes, exemplified by the PEPC gene families in *M. crystallinum* and *K. blossfeldiana*, are encoded as small multigene families of which only one or two CAM-specific isoforms become highly induced during CAM induction to accommodate high rates of nocturnal CO$_2$ fixation (Cushman et al., 1989; Gehrig et al., 1995). In contrast, the expression of the C$_4$ isoforms, which fulfill various anaplerotic housekeeping’ or tissue-specific functional roles, remains essentially unchanged. Multigene families having at least one CAM-specific isogene have also been reported for NADP-ME, NADP-MDH, enolase and PGM in *M. crystallinum* (Cushman, 1992, 1993; Forsthoefel et al., 1995a,b) and for the V-ATPase c subunit genes in *M. crystallinum* (Tsiantis et al., 1996) and *K. daigremontiana* (Bartholomew et al., 1996). Induction of V-ATPase likely takes place to sustain adequate rates of malate transport into the vacuole (Ratajczak et al., 1994; Barkla et al., 1995). Although the exact mechanism of gene recruitment to fulfill CAM-specific functions remains unknown at present, one scenario is gene duplication followed by gradual alterations in the 5’ and 3’ flanking regions and in coding regions which result, ultimately, in the creation of C$_4$ or CAM isoforms, each with distinct expression patterns. Alternatively, recruitment of a specific gene family member may have evolved through recombination or transposon-induced translocation events (Kloeckener-Gruissem and Freeling, 1995). The second class of inducible transcripts is represented by single genes which can apparently play roles in both C$_4$ photosynthesis and CAM as is the case of NAD-GAPDH (Östrem et al., 1990) and PPDK (Füllthaler et al., 1995). For these genes, transcriptional activation under stress conditions seems to occur through the action of stress-responsive enhancer elements.
Chapter 23 Crassulacean Acid Metabolism Induction

For enzymes encoded by multigene families, the relative contribution to CAM of gene products arising from specific family members needs to be determined, particularly when these proteins reside in different subcellular compartments. For example, both mitochondrial NAD-ME, and cytosolic NADP-ME are represented by multiple isoforms (Saitou et al., 1994, 1995; Cook et al., 1995) and enzymes from both compartments are coordinately induced to support daytime malate decarboxylation (Holtum and Winter, 1982; Cushman, 1992; Ingram and Smith, 1995). Likewise, a cytosolic NAD-MDH and a chloroplastic NADP-MDH participate in nocturnal malic acid formation (Holtum and Winter, 1982; Cushman, 1993). Comparative studies of the expression, structure and kinetic properties of isozymes are needed to resolve the role that individual enzymes play in CAM.

V. Regulation of CAM Gene Expression

The switch from $C_3$ photosynthesis to CAM serves as an important paradigm for complex adaptations of photosynthetic carbon fixation to environmental stress. The kinetics of CAM induction depend on the type and severity of the stress and the developmental status of the plant (Cushman et al., 1990; Piepenbrock and Schmitt, 1991; Herppich et al., 1992; Schmitt and Piepenbrock, 1992a). Considering the correlation between stress severity and CAM induction, the buildup of many CAM-related enzyme activities during the transition (Holtum and Winter, 1982; Winter et al., 1982) is directly tied to increases in enzyme protein levels (Winter et al., 1982; Michalowski et al. 1989a; Schmitt et al., 1989) arising from de novo protein synthesis (Foster et al., 1982; Höfner et al., 1987). Transport processes at the tonoplast are enhanced during the CAM switch as a result of increased expression of V-ATPase subunits (Lüttge et al., 1995; Dietz and Arbingter, 1996; Löffel et al., 1996; Tsiantis et al., 1996). Light-dependent chloroplast pyruvate transport is also induced in *M. crystallinum* (Kore-eda et al., 1996), presumably as a result of increased synthesis of the pyruvate transporter. Chloroplastic starch degradation enzymes (Paul et al., 1993) and presumably triose and hexose phosphate transporters are also enhanced during CAM (Neuhaus and Schulte, 1996). Transcriptional, post-transcriptional, and translational controls are primarily responsible for the build-up of CAM-related enzymes, whereas post-translational controls mediate circadian regulation of enzyme activities during the day-night cycle.

A. Transcriptional Regulation

The expression of CAM-specific genes is regulated primarily at the level of transcription. PEPC mRNA accumulation occurs within 2–3 hours following...
salinity or dehydration stress of detached leaves of *M. crystallinum* (Schmitt, 1990) or *K. blossfeldiana* (Brulbert et al., 1993; Taybi et al., 1995). Protein accumulation begins eight to ten hours after stress. Water stress increases PEPC activity, protein and mRNA accumulation even in the youngest leaves of *P. camptotricha* and *P. scandens*, (Ting et al., 1996) and PEPC transcripts in *M. crystallinum* leaves as young as one week old (Schmitt et al., 1996). Run-on transcription assays with nuclei isolated from leaves of *M. crystallinum* have shown that increased mRNA accumulation of many genes is a direct consequence of increased transcription rates (Fig. 1). Transcription rates increase from two- to six-fold in response to salinity stress depending on the gene in question (Cushman et al., 1989; Cushman, 1992, 1993; Vernon et al., 1993; Forsthöfel et al., 1995a,b). Similar increases in transcription rates of a CAM-specific isogene of PEPC have been found in *K. blossfeldiana* induced by short-day photoperiods (N. Richard and J. Brulbert, unpublished).

Transcriptional activation of CAM-specific genes is likely to be mediated by the action of transcriptional activator and repressor proteins through interactions in the promoter regions of these genes. Nuclear extracts from *M. crystallinum* contain multiple DNA-binding proteins which interact with the 5′ flanking region of *Ppc1* to form DNA-protein complexes in vitro over a region of about 1200 nucleotides (Cushman and Bohnert, 1992). One particularly abundant complex, designated PC AT-1, interacts with AT-rich sequences between –205 to 1–28 of the *Ppc1* promoter and displays either increased abundance or tighter DNA binding affinity in nuclear extracts from CAM-induced, salt-stressed plants (Cushman and Bohnert, 1992). Although the exact role of PCAT-1 is unclear, it shares characteristics of HMG-like proteins suggesting it may play an architectural role in the assembly of active transcription complexes during CAM induction (Grasser, 1995). Less abundant DNA-binding complexes, detected in nuclear extracts prepared from roots and leaves of well-watered plants, may function as transcriptional repressors (Cushman and Ho, 1995), light (Terzaghi and Cashmore, 1995), and ethylene (Xu et al., 1996). Comparison of the *Ppc1* and *GapC1* distal regions sufficient for salt-inducible gene expression revealed common *cis*-acting regulatory elements in the 5′ flanking regions of stress-inducible CAM genes (Schaeffer et al., 1995). Fusion constructs consisting of either the *Ppc1* (–977 to –679) or the *GapC1* (–735 to –549) ‘salt-enchancer’ region to their respective non-salt-responsive, ‘minimal’ promoters (–119 or –108, respectively) confirm that these regions are sufficient to confer salt-inducible gene expression. In fact, these regions in isolation are more effective than the intact promoters in conferring salt-induced gene expression, confirming the presence of silencing elements elsewhere in the 5′ flanking regions. Fusion of the *Ppc1* –977 to 6–79 region to a truncated ‘minimal’ (–45) *Ppc1* promoter, however, completely abolished salt-inducibility supporting the requirement for cooperation between at least two *cis* elements for salt-responsive transcription (H. J. Schaeffer and J. C. Cushman, unpublished). Cooperation of two or more *cis* elements for regulation of gene transcription has been documented for promoters responsive to gibberellic acid (Rogers et al., 1994), ABA (Shen and Ho, 1995), light (Terzaghi and Cashmore, 1995), and ethylene (Xu et al., 1996). Comparison of the *Ppc1* and *GapC1* distal regions sufficient for salt-inducible gene expression revealed common MYB consensus binding sites suggesting that MYB-like factors may be involved in controlling transcriptional activation events during CAM induction (Schaeffer et al., 1995). The participation of myb-related homologs from *Arabidopsis* (Urao et al., 1993) and maize (Hattori et al., 1992) in transcriptional responses to ABA, salt and dehydration
stress, suggests that related factors in *M. crystallinum* may also play a role in the expression of inducible CAM gene expression.

In addition to osmo-responsive elements, the promoter regions of CAM-specific PEPC, NAD-GAPDH, and PPDK genes contain GT motifs (BoxII) which may function in light-responsive gene expression and consensus ABA response elements which may participate in ABA-mediated gene expression events (Cushman et al., 1993; Schmitt et al., 1996). Other transcriptional control elements and factors conferring tissue-specific, diurnal or circadian, developmental, dehydration-, light-, and hormone-responsive gene expression must also be present.

**B. Posttranscriptional and Translational Regulation**

Posttranscriptional and translational mechanisms also play a role in regulating CAM expression, however, only indirect evidence for these control mechanisms is presently available. A comparison of transcription rates and transcript accumulation in *M. crystallinum* undergoing CAM induction at different ages showed that *Ppc1* mRNA stability is enhanced in older plants (Cushman et al., 1990). In contrast, *RbcS* transcripts encoding the small subunit of Rubisco decline rapidly upon salt stress (DeRocher and Bohnert, 1993; Bohnert et al., 1997). Transcription rates and mRNA accumulation for enolase increase in response to salinity stress without a corresponding increase in protein amounts suggesting that posttranscriptional/translational control factors participate in the expression of this glycolytic enzyme (Forsthoefel et al., 1995a). An increase in chloroplast RNA-binding proteins that may function to stabilize specific transcripts has been shown to accompany CAM induction (Breiteneder et al., 1994). While changes in mRNA populations following stress have been well documented (Ostrem et al., 1987), changes in mRNA utilization and translational efficiency are likely to contribute significantly to CAM-related expression patterns. During the transition from *C₃* to CAM, total protein synthesis declines, whereas the synthesis of specific proteins, such as PEPC, increases (Höfner et al., 1987). Altered translational activity is correlated with changes in mRNA distribution on polysomes (Bohnert et al., 1999 and enhanced expression of a ribosome-inactivating protein (RIP) in *M. crystallinum* (Rippmann et al., 1997). RIPs may alter translation through the turnover of ribosomes or sub-populations of ribosomes in response to stress.

**C. Posttranslational Regulation**

Posttranslational control mechanisms regulate diurnal or circadian activities of several key CAM enzymes. Most studies have focused on the reversible day/night regulation of PEPC which must be tightly controlled in order to avoid futile cycles of carboxylation and decarboxylation. The activity of this enzyme is influenced allosterically by both positive (glucose 6-P, triose-P) and negative (L-malate) effectors (Chollet et al., 1996; Vidal and Chollet, 1997). In addition, control of PEPC occurs by covalent modification through reversible phosphorylation by PEPC kinase first described in *Bryophyllum fedtschenkoi* and *Kalanchoë blossfeldiana* (Nimmo et al., 1984, 1986; Brulfert et al., 1986) and more recently in *M. crystallinum* (Baur et al., 1992; Li and Chollet, 1994) as well as in numerous *C₃* and *C₄* species (Chollet et al., 1996; Vidal and Chollet, 1997). In CAM plants, the dephosphorylated ‘day form’ of the enzyme is more sensitive to malate inhibition. The more active, phosphorylated ‘night form’ has higher affinity for PEP and is more sensitive to glucose 6-P and triose-P (positive effectors), but less sensitive to L-malate (negative effector) (Nimmo et al., 1986; Jiao and Chollet, 1991). The phosphorylation site responsible for night activation of the CAM enzyme has been located at a highly conserved serine residue present in the N-terminal region of higher plant polypeptides which is absent from bacterial or cyanobacterial enzymes (Vidal and Chollet, 1997), although additional phosphorylation sites may be present. PEPC kinase activity is dependent on mRNA and protein synthesis and is controlled in a circadian fashion (Carter et al., 1991; 1996). In contrast, protein synthesis-dependent dephosphorylation of PEPC by a protein phosphatase 2A (PP2A) does not appear to be regulated by an endogenous rhythm (Carter et al., 1990). PEPC kinase activity is regulated at the level of translatable mRNA in *B. fedtschenkoi* (Hartwell et al., 1996) with PEPC kinase mRNA being approximately 20 times more abundant at night than during the day. PEPC kinase activity disappears within three hours of the decline in translatable mRNA indicating
rapid protein turnover (Hartwell et al., 1996). Inhibition of translation also blocks the appearance of translatable PEPC kinase mRNA indicating that upstream signaling events, such as the operation of the circadian clock, also require protein synthesis (Hartwell et al., 1996). These observations confirm that transcriptional regulatory events can not only govern the production of many CAM enzymes, but also control their post-translational regulation.

PEPC kinase is induced coincidently with its target substrate by salt stress in _M. crystallinum_ (Li and Chollet, 1994). Coordinate induction of PEPC and its regulatory kinase by ABA has been documented in detached leaves of _K. blossfeldiana_. Kinase accumulation is slightly delayed with respect to PEPC synthesis (T. Taybi and J. Brulftert, personal communication). Although the factors controlling PEPC kinase expression or activity are not understood, fluctuations in cytosolic malate levels or the processes that control malate movement into and out of the vacuole appear to play a role in the generation and regulation of its circadian rhythm (Anderson and Wilkins, 1989c). In C₄ plants, however, PEPC kinase appears to be regulated by phosphorylation via an upstream Ca²⁺-dependent protein kinase (Giglioli-Guivarac’h et al., 1996). Molecular cloning of the gene encoding PEPC kinase should greatly facilitate identification of the circadian oscillator and the intracellular signaling mechanisms by which it is regulated (see next section). Progress towards this goal has been made through the partial purification of a Ca²⁺-independent kinase identified as two polypeptides (39 and 32 kDa) capable of phosphorylating purified PEPC from _C₃_, _C₄_, and CAM species. Whether the two proteins represent two isoforms or are the result of proteolytic processing of a single protein remains unclear (Li and Chollet, 1994).

In addition to PEPC, several other CAM enzymes undergo post-translational regulatory phosphorylation events. PEPKCK is phosphorylated in _C₃_, _C₄_, and CAM plants (Walker and Leegood, 1996). In PEPC-type CAM plants, this decarboxylase should only be active during the day to avoid a futile carboxylation cycle between PEP and OAA since both PEPC and PEPCCK are cytosolic. In _Tillandsia fasciculata_, PEPC is phosphorylated at night and dephosphorylated during the day (Walker and Leegood, 1996). This phosphorylation pattern is likely to modulate the activity of the enzyme over the course of the diurnal cycle, however, it is not known how this occurs or whether PEPCCK is regulated by light or in response to a circadian rhythm.

Diurnal and circadian regulation of nitrate reductase in _B. fedtschenkoi_ is likely to involve multi-step post-translational regulation (Huber et al., 1996; Lillo et al., 1996). Covalent modification of NAD-ME may also be responsible for diurnal changes in the kinetic properties of this enzyme (Cook et al., 1995). Post-transcriptional control of enolase activity by reversible phosphorylation has been suggested for enolase from _M. crystallinum_ (Forsthoefel et al., 1995a). Likewise, the activities of many chloroplastic and possibly cytosolic enzymes such as NAD-GAPDH, NADP-MDH, FBP, and enolase are light-regulated through covalent redox-modification of the ferredoxin-thioredoxin system which acts at redox-sensitive cysteine residues that participate in disulfide bridge formation (Anderson et al., 1995).

### D. Circadian Rhythms

The circadian rhythm of photosynthetic gene expression in plants has provided important insights in the mechanisms of circadian clocks (Anderson and Kay, 1996). One of the most extensively studied circadian rhythms includes the rhythm of CO₂ metabolism in _Bryophyllum_ (_Kalanchöe_) _fedtschenkoi_ (Wilkins, 1992) and _K. daigremontiana_ (Lüttge and Beck, 1992, 1996; Lüttge et al., 1996). Studies of CAM behavior in _K. blossfeldiana_ (Queiroz and Morel, 1974), _K. fedtschenkoi_ (Wilkins, 1984), and _K. daigremontiana_ (Buchanan-Bollig and Smith, 1984a,b) have shown that plants kept under constant environmental conditions exhibit a persistent circadian rhythm of CO₂ exchange that is directly related to the circadian rhythm of PEPC activity and properties (Nimmo et al., 1984). Changes in enzyme properties parallel the in vitro and in vivo phosphorylation state of PEPC and PEPC kinase activity (Nimmo et al., 1986, 1987; Carter et al., 1991).

The reversible day/night pattern of PEPC phosphorylation is not simply controlled by light as PEPC kinase activity appeared several hours after the onset of darkness and disappeared a few hours before the end of darkness (Nimmo et al., 1984; Carter et al., 1991). Furthermore, the phosphorylation state of PEPC exhibits a persistent circadian rhythm under conditions of continuous illumination or darkness (Nimmo et al., 1987; Kusumi et al., 1994). Extremes in light intensity and temperature perturb circadian rhythms of CO₂ fixation (Anderson and
Wilkins, 1989a,b,c; Lütting and Beck, 1992) through modifications not only of PEPC kinase activity (Carter et al., 1995a,b; 1996), but also through changes in malate concentrations in the vacuole and cytoplasm as controlled by permeability or transport at the tonoplast (Grams et al., 1996, 1997). These observations suggest that malate also plays a role in regulating PEPC kinase activity and/or PEPC activity. PEPC kinase activity can also be disrupted by treating detached leaves with inhibitors of transcription (cordycepin) and translation (cycloheximide or puromycin) (Carter et al., 1991; Nimmo, 1993; Hartwell et al., 1996). Despite these observations, the origin and the nature of the circadian oscillator controlling PEPC kinase activity remain elusive. Recent observations that PEPC kinase expression exhibits a circadian rhythm controlled at the level of transcription and possibly also at the level of translation (Hartwell et al., 1996), strongly suggest that PEPC kinase is an important component of the circadian oscillator. Protein phosphatase inhibitors are known to disrupt circadian oscillator function (Comolli et al., 1996). This observation provides further support for the role of protein phosphorylation in controlling the circadian rhythm of CAM. Once the gene for PEPC kinase is cloned, it should be possible to resolve whether this kinase is generating oscillations or controlling them. Entrainment studies using Bryophyllum leaves have confirmed that phytochrome is likely to serve as the only photoreceptor for the regulation of the circadian rhythm (Harris and Willkins, 1978a,b). The input and output pathways of many phytochrome regulated circadian clocks utilize Ca^{2+} as a second messenger (Johnson et al., 1995; Anderson and Kay, 1996). Knowledge of the biochemical components of the circadian clock should lead to insights into the signaling events controlling clock entrainment and the induction of phase shifts.

**E. Tissue-Specific Regulation**

Traditionally, CAM was thought to be present in all chloroplast-containing tissues and absent in hydrenchymous tissues without developed chloroplasts as indicated by differences in CAM enzyme distribution between photosynthetically active and water-storing tissues (Kluge and Ting, 1978; Earnshaw et al., 1987; Winter, 1987; Springer and Outlaw, 1988). In general, chloroplast-containing cells fit the classical CAM enzyme spectrum, whereas chlorophyll-deficient tissues express CAM weakly suggesting they serve in a ‘CO_{2} storage’ capacity (Springer and Outlaw, 1988). However, recent analyses using histochemical techniques have established distinct spatial separation of CAM components within tissues. The most exhaustive studies of cell specialization within tissues have used *Peperomia campotricha* (Nishio and Ting, 1987). In this species the leaf is organized into four cell layers: an upper multiple epidermis, a middle one- to two-layered palisade mesophyll, a lower spongy mesophyll, and an abaxial epidermis. Physical separation of these tissues indicated that CAM enzyme activity (PEPC and ME) and nocturnal CO_{2} fixation predominates in the spongy mesophyll and to a lesser extent to the multiple epidermis (Nishio and Ting, 1987). Tissue printing of *Peperomia* leaves confirmed PEPC protein and transcript expression localized preferentially to these two layers (Ting et al., 1994). In contrast, enzymes characteristic of light-dependent C_{3} photosynthesis and Rubisco activity are more abundant in palisade mesophyll (Nishio and Ting, 1987, 1993). Rubisco protein is distributed throughout the leaf whereas *rbcS* mRNA is most abundant in the palisade layer (Ting et al., 1994). *Peperomia* species may be unique in the degree of tissue-specific distributions of CAM and C_{3} enzymes, however, in view of the morphological complexity of leaves in many CAM plants, a variety of tissue-specific expression patterns of C_{3} and CAM enzymes may exist.

Tissue differentiation determines the extent to which CAM is induced. Photoperiodic (short-day) induction of CAM is more effective in leaves than in callus tissue from *K. blossfeldiana* (Brulfert et al., 1987) with the degree of induction being correlated positively with the degree of tissue organization (Kluge et al., 1987). Likewise, salinity stress has little effect on malate accumulation and PEPC activity or expression in heterotrophic cell suspension cultures (Treichel et al., 1988; Thomas et al., 1992) or green callus tissues from *M. crystallinum* (Yen et al., 1995) compared to the inductive effects observed in intact plants. The basis for the apparent dependence of the CAM syndrome on organized tissues is not fully understood.

The inability to induce CAM in cell cultures may be due to the lack of a functional photosynthetic apparatus (Thomas et al., 1992a). Recent studies with photomixotrophic cell cultures of *M. crystallinum* grown on starch showed only a weak (four-fold) induction of PEPC activity (Yen et al., 1995), although this was greater than that observed for
heterotrophically grown cell suspensions. These results suggest that the development of a photosynthetic machinery is paralleled by an increase in CAM-related gene expression. However, photoautotrophic cell cultures from *M. crystallinum*, grown with CO₂ as the sole carbon source, were unable to fully develop CAM (Willenbrink and Huesemann, 1995). These cells showed significantly increased rates of CO₂ fixation in the light, but when salt stressed, did not display CAM-related fluctuations or net nocturnal accumulations of malic acid. Nonetheless, they exhibited slight increases (1.5- to 4-fold) in extractable PEPC, NADP-ME, NAD- and NADP-MDH, and PPDK activities (Willenbrink and Huesemann, 1995). These increases could signal partial CAM induction in response to salinity at the cellular level without the corresponding fluctuations in C₄ acid or starch accumulation. Such fluctuations may depend on regulatory signals that are disrupted in the suspension cell cultures which may lack fully functional chloroplasts. One of the most important factors governing photosynthetic gene expression is the metabolic flux of carbohydrates and other metabolites, specifically hexoses, within sink and source leaves (Koch, 1996). In isolated maize protoplasts, the expression of several light-regulated photosynthetic genes, including PEPC and other chloroplast components such as the phosphate/triose-phosphate translocator is repressed by feedback inhibition of hexose sugars (Sheen, 1990, 1994). Hexokinases may serve as sensors and signaling molecules for sugar repression of photosynthetic genes (Jang and Sheen, 1997; Jang et al., 1997). Thus, it is likely that the signaling mechanisms associated with carbohydrate metabolism are disrupted under tissue culture conditions preventing appropriate expression and regulation of CAM-specific enzymes.

### VI. Signal Transduction

The mechanisms by which stimuli are transduced into the activation of CAM gene expression and other stress-adaptive responses are poorly understood. Factors modulating gene expression in response to development, environmental stress, and plant growth regulator homeostasis reveal complex signal-transduction pathways for CAM induction. Following the initial perception of external stimuli by sensor/receptor complexes, signaling molecules (e.g., ABA) or second messengers (e.g., Ca²⁺) form a complex web of parallel and converging pathways (Shinozaki and Yamaguchi-Shinozaki, 1996). Glimpses into the complexity of this web have already been obtained in *M. crystallinum* indicating multiple signal transduction pathways leading to CAM induction and other stress adaptations (Fig. 2) (Vernon et al., 1993; Tsiantis, 1996).

Among the well-characterized genes and proteins for stress-responsive pathways that assure survival for *M. crystallinum* by metabolic adaptations are three genes in different pathways. *Ppc1*, the gene encoding an induced isoform of PEPC, is strictly related to CAM biochemistry (Cushman et al., 1989), whereas *Vmacl* (*Atpvc*), which encodes the small c subunit of the vacuolar H⁺-ATPase, is responsible for pH and proton gradient maintenance across the tonoplast membrane (Löw et al., 1996; Tsiantis et al., 1996). The third gene is *Imt1*, which encodes inositol-O-methyltransferase, an enzyme involved in biosynthesis of ononitol (Vernon and Bohnert, 1992; Ishitani et al., 1996). Although all three genes are induced by salinity, they exhibit very different expression patterns in response to other types of environmental stress, reflecting distinctive aspects of their specific roles in stress adaptation (Vernon et al., 1993; Bohnert and Jensen, 1996; Tsiantis, 1996; Tsiantis et al., 1996). *Ppc1* mRNA accumulation can be readily induced by drought and treatment with 6-BAP, but not by low temperature. In contrast, *Imt1* mRNA fails to accumulate in response to drought and 6-BAP treatment, but does accumulate in response to low temperature (Vernon et al., 1993). Salinity induction of *Vmacl* expression appears to rely specifically on ionic stress, but not on osmotic stress components as neither mannitol nor drought are particularly effective inducers in mature plants (Tsiantis, 1996). Additional complexity is introduced by development because *Ppc1* and *Vmacl* cannot be induced in very young plants, whereas *Imt1* is only salt-inducible in mature plants, but is equally drought and salt-inducible in cotyledons, and strongly salt- and marginally drought-inducible in seedlings and very young plants (Tsiantis, 1996). Crosstalk between different signal-transduction chains can give rise to signal amplification and a wide variety of complex responses.

#### A. Photoreception

Phytochrome plays a central role in the initiation of...
light-sensing signal transduction pathways (Quail et al., 1995). Phytochrome participates not only in the photoperiodic induction of CAM in K. blossfeldiana (Gregory et al., 1954; Brulfert et al., 1975; Brulfert et al., 1985) and the appearance of a CAM-specific isoform of PEPC (Brulfert et al., 1982a,c), but also regulates the circadian rhythm of CO$_2$ metabolism in Bryophyllum (Harris and Wilkins, 1978a,b). In M. crystallinum, long photoperiods, high light intensity, and red light enhanced NADP-ME and PEPC expression (Cheng and Edwards, 1991; McElwain et al., 1992; Cockburn et al., 1996). A low, but not a high, ratio of red:far red light stimulated PEPC expression and malate accumulation (Cockburn et al., 1996). These effects, which were amplified in salt stressed plants, suggested that phytochrome and NaCl or ABA may act along the same or parallel signal transduction pathways to promote CAM induction (McElwain et al., 1992; Cockburn et al., 1996). Recent studies based on microinjection of putative signaling molecules or inhibitors indicated that heterotrimeric G proteins participate in three distinct phytochrome signal transduction pathways (Neuhaus et al., 1993; Bowler et al., 1994a,b). Phytochrome A activates G proteins leading to increases in cGMP levels or cellular levels of Ca$^{2+}$ and the activation of calmodulin (Millar et al., 1994). The same signaling pathways are likely to both activate and repress different responses (Neuhaus et al., 1997). We expect that similar phytochrome-mediated signal transduction mechanisms participate in the activation or repression of CAM gene expression (see Fig. 3).

During the transition from C$_3$ to CAM, stomatal behavior is reversed, relative to stomatal rhythms in C$_3$ and C$_4$ plants. Red and/or blue light fail to induce stomatal opening in P. afra plants performing CAM (Lee and Assmann, 1992). In well-watered M. crystallinum, blue light is more effective than red light in opening stomata. When in CAM mode, however,
illumination also failed to open stomata suggesting that the blue- and red-light photoreceptors become inoperable (Mawson and Zaugg, 1994). Treatment of epidermal tissues from plants performing CAM with fusicoccin, a fungal toxin that activates the plasmalemma H⁺-ATPase, failed to cause an increase in stomatal opening, whereas ABA-induced stomatal closure was still functional. Inactivation of guard-cell photoreceptors may allow other factors, such as ABA or changes in [CO₂], to dictate stomatal movements (Mawson and Zaugg, 1994). The nature and role of photoreceptors and other components in the signal transduction pathway(s) operating in response to light in CAM plants remain uncertain at
present. However, recent evidence suggests that CAM induction by NaCl stress abolished both the white- and blue-light-stimulated stomatal opening and lightdependent zeaxanthin formation (Tallman et al., 1997). These results suggest that the inhibition of light-dependent zeaxanthin formation in guard-cell chloroplasts may be one of the important components that regulates the shift from diurnal to nocturnal stomatal opening during CAM induction. Other components such as \( \text{Ca}^{2+} \)- or CaM-dependent protein kinases may also be involved in maintaining stomatal closure (Cotelle et al., 1996; Li et al., 1998).

**B. Intracellular Calcium**

In higher plants, intracellular \( \text{Ca}^{2+} \) acts as an important second messenger for signal transduction associated with a wide range of stimuli and plant growth regulators (Poovaiah and Reddy, 1993; Bethke et al., 1995; Bush, 1995). The concentration of \( \text{Ca}^{2+} \) within various subcellular compartments is tightly controlled by a series of channels and pumps which are in turn regulated by extra- or intracellular signals. For example, ABA-, stretch-, or voltage-dependent \( \text{Ca}^{2+} \) channels at the plasma membrane, inositol 1,4,5-triphosphate (IP_3), cyclic ADP-ribose, and voltage-dependent channels at the tonoplast, and voltage-gated (presumably mechanosensitive) channels at the endoplasmic reticulum have been characterized along with \( \text{Ca}^{2+} \)exchangers and \( \text{Ca}^{2+} \) ATPases (Bethke et al., 1995; Bush, 1995; Barbier-Brygoo et al., 1997). A variety of signals cause transient, sustained or oscillating changes in \( \text{Ca}^{2+} \) in different subcellular locations (Bethke et al., 1995; Johnson et al., 1995; Knight et al., 1996). External signals (light, NaCl) and hormones (ABA) involved in CAM, as well as the circadian clock that regulates the daily CAM rhythm, are likely to act through fluctuations of intracellular \( \text{Ca}^{2+} \). However, direct evidence for the participation of \( \text{Ca}^{2+} \)signaling in CAM induction is lacking.

Recent experiments with detached *M. crystallinum* leaves provided indirect evidence for the role of intracellular [\( \text{Ca}^{2+} \)] in CAM induction. Treatment of detached leaves with the \( \text{Ca}^{2+} \) ionophore, ionomycin, and thapsigargin, a specific inhibitor of endomembrane \( \text{Ca}^{2+} \)-ATPases and stimulator of intracellular \( \text{Ca}^{2+} \) release, resulted in increased *Ppc1* and *Vmacc1* mRNA accumulation (Tsiantis, 1996). The effect of thapsigargin on *Ppc1* expression was time-dependent, dose-responsive, and synergistic with NaCl treatment (Taybi and Cushman 1998). These results are confirmed by the observation that the calcium chelator, EGTA, completely abolished *Ppc1* mRNA induction by thapsigargin, salinity, drought, and ABA treatments (Taybi and Cushman, 1998). In contrast, thapsigargin and ionomycin treatments did not induce *Imt1*, suggesting that expression of this gene is regulated by a distinct signal transduction pathway (Tsiantis, 1996).

**C. Protein Kinases and Phosphatases**

Protein phosphorylation and dephosphorylation participate in a wide range of signal transduction pathways (Stone and Walker, 1995; Smith and Walker, 1996). Signal perception is mediated by transmembrane receptors through the activation of a kinase domain intrinsic to receptor protein kinases (RPKs) (Braun and Walker, 1996). Recently, an RPK from *Arabidopsis* was isolated that is induced by dehydration, high salt, low temperature and ABA treatment suggesting that receptor kinases participate in the transmission of ABA and environmental stress signals (Hong et al., 1997). Downstream targets for RPK-mediated signaling have been identified as type 2C protein phosphatases which interact in a phosphorylation-dependent manner with specific subsets of RPKs (Braun et al., 1997). In *Arabidopsis*, water-stress-responsive gene expression is mediated by at least four independent signal transduction pathways with some pathways being ABA-dependent, whereas others are ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 1996). In addition, various signal transduction components such as MAP kinases, two-component histidine kinases, calcium-dependent protein kinase, and phospholipase C are stress-induced suggesting that both IP_3,\( \text{Ca}^{2+} \) and MAP kinase cascades are involved (Jonak et al., 1996; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997; Mizoguchi et al., 1997). These observations suggest that water stress stimuli that lead to CAM induction are mediated by multiple, independent signal transduction pathways involving protein phosphorylation.

Specific inhibitors of kinases and phosphatases have provided a powerful approach to initially assess the role of protein phosphorylation in controlling the cellular events leading to CAM induction. Okadaic acid, a toxin that inhibits PP2A and to a lesser extent PP1, inhibited the salt-responsive induction of *Ppc1* and *Imt1* transcripts, whereas it was not effective in
abolishing the induction of Vma1 (Tsiantis, 1996). Okadaic acid and the PP2A inhibitor, cantharidic acid, were also effective inhibitors of Ppc1 transcript accumulation induced by dehydration and ABA. These results implicate PP2A (and PP1) or related phosphatases in signaling leading to CAM induction, however, such experimental results must be viewed with extreme caution given the nonspecific effect of these inhibitors. Cyclosporin A, which specifically targets Ca\(^{2+}\)/calmodulin-activated PP2B (calcineurin), blocked the salt-induction of Imt1, but did not negatively affect Ppc1 or Vma1 expression (Tsiantis, 1996). Cyclosporin A, in fact, resulted in increased Ppc1 transcript accumulation during stress or ABA treatments of detached leaves (Taybi and Cushman, 1998). Thus, PP2B (calcineurin) activity seems to participate in negative or positive regulation of both ionic and dehydration stress signaling depending on the specific gene in question.

Although pharmacological studies cannot rigorously identify specific kinases or phosphatases, they clearly indicate that multiple independent, yet interrelated signal transduction pathways lead to CAM gene expression (Fig. 2). An example of convergent signaling pathways was revealed by treating detached M. crystallinum leaves with W7, a specific inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinases, which blocked Ppc1 transcript accumulation in response to ionic, osmotic, and dehydration stress (Taybi and Cushman, 1998). This result suggests that Ca\(^{2+}\)/CaM protein kinases play a role in stress-induced increases in Ppc1 expression. In contrast, the effect on CAM induction in M. crystallinum was enhanced by cyclosporin A pretreatment presumably through the opposing activities of PP2B and Ca\(^{2+}\)/CaM kinase. Interestingly, both activities can be influenced by intracellular [Ca\(^{2+}\)]. Differential sensitivity to changing intracellular [Ca\(^{2+}\)] through different Ca\(^{2+}\)-dependent kinases and phosphatases each having unique Ca\(^{2+}\)-binding properties and kinetics could explain selective activation of different signaling pathways in response to various stimuli. Definitive identification of the specific kinases and phosphatases and branchpoints associated with these different pathways will require a combination of biochemical and genetic approaches.

Characterization of sensors and transducers will be essential to advance our understanding of the various signaling pathways leading to CAM induction. Such analyses are presently underway in M. crystallinum from which a number of protein kinase genes have been cloned. One ser/thr protein kinase cDNA termed MK9 (Baur et al., 1994) shares similarity to an ABA-, dehydration-, cold-, and osmotic stress-inducible protein kinase from wheat (Holaapa and Walker-Simmons, 1995). MK9 also shares sequence homology with SNF1 (‘sucrose nonfermenting’), a protein kinase from yeast required for derepression of glucose-repressed genes (Jiang and Carlson, 1996) that may act downstream of hexokinase in sugar signaling (Jang and Sheen, 1997). MK9 expression is enhanced by drought stress, ABA and 6-BAP treatment (J. Cushman, unpublished) suggesting it may play roles in either sugar-responsive or stress-responsive signaling or crosstalk between these pathways. A second ser/thr protein kinase cDNA (MK6) has been described which shows preferential expression at night in M. crystallinum plants performing CAM (B. Baur, personal communication). This protein kinase may play a role in controlling the diurnal or circadian expression of CAM enzymes. Additional protein kinases related to cyclic-nucleotide-dependent (PKA and PKG) or Ca\(^{2+}\)- phospholipid-dependent kinases (PKC) have also been isolated from M. crystallinum (‘AGC’ kinases, Table 1). This group of kinases is likely to act downstream of second messengers in signal transduction cascades (Stone and Walker, 1995). Other signal transducing components such as Ca\(^{2+}\)-dependent kinases, G-proteins and 14-3-3 proteins have also been characterized in M. crystallinum (Table 1).

D. Plant Growth Regulators

Induction of CAM gene expression has been associated with the action of several plant growth regulators, although their precise function remains unclear. ABA acts through a complex network of signaling pathways (Giraudat, 1995). Endogenous increases in ABA or exogenous ABA application correlate with CAM induction and CAM gene expression (Sipes and Ting, 1985; Ting, 1985, 1987; Chu et al., 1990; Thomas et al., 1992b; Thomas and Bohnert, 1993; Dai et al., 1994; Taybi et al., 1995). The duration of ABA application dictates the extent of CAM induction (Taybi et al., 1995). However, ABA has never been shown to induce Imt1, whereas it will induce Vma1, and Ppc1 (depending on age) transcript accumulation (Tsiantis, 1996).
To determine if ABA exerts its action directly or indirectly through reduced CO$_2$ concentrations, detached _K. blossfeldiana_ leaves were exposed to ABA under normal air or CO$_2$-free conditions (Fig. 3A). A rapid increase in stomatal resistance in air was observed, however, stomatal closure was delayed significantly in the absence of CO$_2$ (Fig. 3A). ABA elicited an increase in PEPC activity (Fig. 3B) and malate consumption (Fig. 3C) which was counteracted by low CO$_2$. ABA and CO$_2$ deficit do not act synergistically with respect to changes in PEPCase activity or malate accumulation. Furthermore, CO$_2$ deficit does not trigger CAM induction brought about by either exogenous ABA exposure or environmental stress. Conversely, when detached leaves are supplied with ABA in the presence of saturating CO$_2$ (5%), a typical CAM pattern of CO$_2$ exchange is observed at night, confirming that ABA control of stomatal behavior is not governed by [CO$_2$] (Taybi, 1995).

Cytokinins regulate various developmental processes, however, little is known about their role in signal transduction (Binns, 1994). Cytokinins, like ABA, induce a variety of CAM-related transcripts (Table 1), mimic salinity- or drought-induced CAM induction (Thomas and Bohnert, 1993; Dai et al., 1994), and mimic salt-induced physiological reactions such as proline, polyol, and osmotin accumulation when applied to roots (Thomas and Bohnert, 1993; Dai et al., 1994). However, cytokinins can also have an inhibitory effect on salinity- or dehydration-induced PEPC expression when supplied to detached leaves (Schmitt and Piepenbrock, 1992b; Dai et al., 1994). Recently, a histidine RPK (Kakimoto, 1996) and cytokinin-responsive SNF1-like kinases have been identified (Sano and Youssefian, 1994). Characterization of related components in CAM plants should lead to a greater understanding of cytokinin signaling mechanisms during CAM induction.

**VII. Future Prospects**

CAM is a complex adaptation to CO$_2$- or water-limited environments requiring the coordination of gene expression, biochemistry, physiology, and anatomical features that distinguish the pathway from C$_3$ photosynthesis. Recent work with facultative CAM species has begun to yield substantial insights into how developmental and environmental signals initiate CAM gene expression and maintain circadian oscillations of the cycle. To further advance our knowledge of the molecular basis of CAM induction, a combination of biochemical, genetic and molecular approaches will be needed.

**A. Genetic and Molecular Genetic Analysis of CAM**

Mutant collections in genetic model plants such as maize (Neuffer et al., 1996) and *Arabidopsis* (Meyerowitz and Somerville, 1994) have provided plant biologists with essential tools for understanding plant structure, development, and metabolism. To our knowledge, however, there are no reports of mutants defective in CAM or of mutants in any CAM species. Facultative CAM species, such as *K. blossfeldiana* or *M. crystallinum*, will be particularly useful if they can be developed into genetic models. *M. crystallinum* is a logical choice since it represents the best-studied CAM plant at the molecular level (see Table 1). It is a self-fertile species with a relatively small genome, twice that of *Arabidopsis*, and nine chromosomes (2N = 18), and it shows developmentally regulated polyploidy (DeRocher et al., 1990; Adams et al., 1997). The normal life cycle of *M. crystallinum* takes five months to complete in its natural habitat (Winter et al., 1978). However, the ability to grow the plants under long photoperiods, which shortens the life cycle to only seven weeks, miniaturizes the plants, and accelerates CAM induction (Cheng and Edwards, 1991), will expedite genetic studies.

Strategies which have been used to isolate photosynthetic carbon metabolism mutants (Somerville, 1986) can be applied to detect mutants in CAM species. One simple strategy involves screening of mutagenized plants for the presence or absence of starch (Caspar et al., 1985) which, for example, led to the detection of a mutant that lacked the chloroplast isozyme of phosphoglucomutase. Mutations in Rubisco activase and Rubisco enzymes have been obtained by growing mutagenized plants at elevated CO$_2$ concentrations (0.7–1 %) followed by transfer to a low CO$_2$ atmosphere (Somerville, 1984; Blackwell et al., 1988; Lea and Forde, 1994). Mutants lacking C$_4$ photosynthetic pathway enzymes, such as PEPC, NAD-ME, and glycine decarboxylase have been described (Dever et al., 1995). In addition, several maize C$_4$ photosynthetic mutants have been
characterized that are defective in bundle sheath and mesophyll cell differentiation (Langdale and Kidner, 1994; Langdale, 1995; Roth et al., 1996).

To begin genetic studies in a CAM species, mutant collections have been established in *M. crystallinum* following irradiation with fast neutrons and exposure to chemical mutagens (H. J. Bohnert and J. C. Cushman, unpublished). Pilot experiments using both X-ray- and EMS-generated mutants have resulted in the isolation of phenotypes that should be meaningful for the genetic dissection of CAM. However, T-DNA (Feldman, 1991) or transposon mutagenesis strategies (Altmann et al., 1995) and promoter and exon trapping approaches (Smith and Fedoroff, 1995) should also be explored. Selection schemes for screening putative CAM mutants that rely on detecting plants that fail to conduct nocturnal acidification and/or daytime starch accumulation have also been developed (Fig. 4). A concerted effort by several laboratories for generating, characterizing, and maintaining CAM mutant populations should provide an important genetic resource that will aid in the identification and characterization of genes important to CAM.

Another strategy that will allow rapid identification of novel genes from CAM plants is partial or complete sequencing of cDNAs for expressed sequences. Such expressed sequence tags (ESTs) complement genetic analyses and provide markers for genomic mapping and gene expression studies. Analysis of large numbers of randomly selected cDNAs from rice (Uchimiya et al., 1992; Sasaki et al., 1994), maize (Keith et al., 1993), *Brassica napus* (Park et al., 1993), and *Arabidopsis* (Cooke et al., 1996) provided an invaluable resource for plant molecular biologists and for genome sequencing efforts. As a prelude to the analysis of the *M. crystallinum* genome, two EST projects have been initiated: one targeting leaf tissue of unstressed and stressed plants (J. C. Cushman, unpublished), and the other targeting transcripts specific for epidermal bladder cells (D. E. Nelson and H. J. Bohnert, unpublished) that seem to be essential for ion homeostasis under salt stress conditions. Rather than representing a duplication of ongoing EST sequencing programs in other species, the judicious sequencing of cDNAs in a CAM species is justified because CAM is a unique adaptation absent from *C₃* or *C₄* model plants.

**B. Transgenic Analysis**

Central to a long-term strategy for developing any CAM plant into a useful genetic model system is the ability to easily and rapidly transform the species. Several species exhibit susceptibility to Agro-

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![Fig. 4. *M. crystallinum* plants from mutagenized by EMS or fast neutron bombardment are planted in soil and screened after salinity stress treatment for the ability to perform nocturnal acidification or daytime starch accumulation. Duplicate leaf disc punches are collected at dawn or at dusk in a 48-well microtiter plate. At dawn, leaf discs are assayed for pH using chlorophenol red, a pH color indicator for the pH range spanning 5.0 (yellow, indicated by white circles) to 6.0 (red, indicated by black circles) to monitor *C₄* acid accumulation. Intermediate levels of acidification are indicated by gray circles. At dusk, leaf discs are screened for starch accumulation using a mixture of iodine and potassium iodide. Wildtype plants that accumulate normal levels of starch show intense iodine staining (indicated by dark circle). Mutants deficient in daytime starch accumulation show weak iodine staining (indicated by white circles). Intermediate levels of starch accumulation are indicated by gray circles. Plants failing one or both tests are scored as putative CAM mutants (indicated by asterisk) and are selected for further characterization.](image-url)
bacterium infection including *K. daigremontiana* (Garfinkel and Nester, 1980; Boulanger et al., 1986) and *M. crystallinum*. Successful *Agrobacterium*-mediated transformation of CAM plants has only been reported for a few species including *K. lacinia* (Jia et al., 1989) and *K. blossfeldiana* (Aida and Shibata, 1996). In *M. crystallinum*, transgenic hairy-root cultures (Andolfatto et al., 1994) or callus tissue (J. C. Thomas, T. Wulan and J. C. Cushman, unpublished) have been obtained following transformation by *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively. Even though fertile *M. crystallinum* plants can be regenerated via organogenesis from tissue explants (Meiners et al., 1991) and callus tissue (Wang and Lüttge, 1994) with low efficiency, no transgenic plants have been reported. Several reporter genes encoding β-glucuronidase (GUS), luciferase (LUC), and synthetic green fluorescence protein (GFP), are expressed in transient expression assays of bombarded *M. crystallinum* leaves and stably transformed tissues (Cushman et al., 1993; Schaeffer et al., 1995; T. Wulan, J. C. Cushman and D. E. Nelson, unpublished), indicating that these proteins provide suitable reporters for transgenic analyses. The ability to efficiently transform model CAM species will allow genetic manipulation of the CAM pathway and should facilitate investigations into the expression, structure, and function of enzymes and other key regulatory components.

Transgenic CAM plants will be essential for analyzing gene expression patterns that are distinct from those found in *C₃* or *C₄* plants. Several *M. crystallinum* promoters, including those for *Ppc1* (Cushman and Bohnert, 1993), *MipA*, *MipB*, *Vmacl*, *Int1*, and *Inps1* (S. Yamada, M. Tsiantis and D. Nelson, unpublished), function in transgenic tobacco and *Arabidopsis* leading to strong expression of reporter proteins. However, in some cases, as with the *Ppc1* promoter, the salt-inducible expression pattern observed in *M. crystallinum* is not retained in transgenic tobacco suggesting that this *C₃* plant lacks the necessary regulatory machinery (via activation or repression) to confer salinity-stress inducible expression (Cushman and Bohnert, 1993). The expression of other CAM-gene promoters in *C₄* plants and the comparative analysis of the constructs in transgenic CAM plants, will provide information about the regulation, inducibility, and cell-specificity of CAM-specific control elements, whose existence, at present, can only be inferred.

**VIII. Conclusions**

The ability of some CAM plants to adapt to environmental stress by altering their ability to conduct CAM represents an important paradigm in photosynthesis research. Over the last eight years, our knowledge of the molecular genetics of CAM has increased tremendously, especially from research with facultative CAM plants. The picture of CAM induction that emerges is complex with gene expression changes being controlled at many different levels and multiple signal transduction pathways participating in the perception of diverse environmental factors. While a handful of stress-induced genes in the CAM pathway have now been characterized, it is anticipated that hundreds of genes and their corresponding gene products will be directly or indirectly involved. New initiatives such as large-scale comparative sequencing efforts of expressed genes and genomic DNA will provide a wealth of new information for molecular phylogenetic studies. The development of CAM mutants and efficient transformation systems promise to usher in a new era of research on CAM. These approaches, coupled with the addition of suitable cell biological systems, based on cell suspension cultures, protoplasts, or microinjection of single cells, will allow a thorough dissection of CAM regulation and signal transduction processes, and will open the door to new and exciting insights into the molecular mechanisms governing CAM induction.

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Summary

The taxonomic and ecological diversity of CAM plants is testament to a suite of morphological and metabolic attributes. These have evolved under contrasting selective pressures in up to 7% of all plant species many times over the past 10–100 Ma. The water and carbon conserving features of CAM impose: i) morphological constraints in terms of the diffusive limitations of succulence in cells or organs and ii) metabolic constraints in terms of maintaining two temporally separated carboxylation systems (usually within the same cell) and a reciprocating pool of carbohydrates which are unavailable for growth. Despite these limitations, the expression of CAM is characterized by a highly plastic response to environmental perturbations which not only permits survival under variable adverse conditions but can also result in high annual productivities of some species. This chapter will consider the biochemical components and physiological consequences of this photosynthetic plasticity. The implications for plant carbon balance, photosynthetic integrity and water use efficiency will be considered in both constitutive and facultative CAM species in response to daily, seasonal and possible future environmental conditions.

I. Introduction: Traditional Understanding and Approaches

Physiological ecologists, in defense of CAM, often invoke the phylogenetic diversity which accounts for perhaps 7% of plant species (Winter and Smith, 1996), and the biochemical convergence which maps onto associated succulent morphological characteristics. We also traditionally seek recourse to the high potential productivities of CAM encountered in some genera (Nobel, 1988). In reality, despite these broader ecophysiological implications, it is the fascination for setting the complexities of the CAM cycle in the context of molecular and biochemical compartmentation which drives forward our research (Chapter 23, Cushman et al.). Here, we hope to demonstrate that lessons from CAM, when set in an environmental context, provide a key to understanding metabolic plasticity and gene expression constrained by diffusive limitations of succulence in cells or organs.

A. CAM: Experimental Approaches and Characterization

Crassulacean acid metabolism presents a challenge to the environmental physiologist, because of the need to monitor biochemical and physiological characteristics throughout a 24 h cycle (Fig. 1). At night, when stomata open, CO₂ uptake, synthesis of organic acids and utilization of carbohydrate reserves allow low rates of transpiration. By day, CO₂ is regenerated from the stored malic acid, usually in sufficient concentrations to close stomata and suppress Rubisco oxygenase and hence photorespiration (Cockburn et al., 1979; Spalding et al., 1979). To quantify the various components of the CAM cycle and integrate metabolic activity across any 24 h period, Osmond (1978) defined four Phases of CAM in terms of net CO₂ exchange, transpiration and stomatal conductance (Fig. 2). Such delineation of CAM can be refined through concurrent measurements of the difference in cell-sap titratable acidity at dusk and dawn (ΔH⁺), since organic acid accumulation during Phase I reflects gross CO₂ fixation (external + internal respiratory CO₂). Thus, ΔH⁺ provides a more direct assessment of the magnitude of night-time CAM activity, although it is often assumed that acid accumulation is not extended into the light period (see below) and that malic acid is not consumed at night to balance energetic requirements for accumulation in the vacuole. During the light period, because CO₂ regeneration is effectively internalized, PS II fluorescence offers great potential to track PS II photon utilization and sink demand (Winter et al., 1990; Roberts et al., 1998).

The day-night cycle is also accompanied by substantial changes in water status and solute concentration. Gas exchange can be used to infer daily, instantaneous patterns of water use efficiency, while the pressure chamber and osmometer remain an essential complement to many studies of CAM. One technique which has successfully been used to infer both the long-term (seasonal) and short-term (daily) changes in CAM activity is the use of carbon-isotope discrimination analysis (Chapter 17, Brugnoli and Farquhar). For constitutive CAM plants, the low discrimination towards ³¹C produces C₄-like organic material and clearly separates CAM material from C₃. However, C₄-CAM intermediates are often not distinguished by this technique (Griffiths, 1992). Measurements of instantaneous carbon-isotope discrimination during leaf gas exchange have provided unequivocal evidence for the transition from C₄ to C₃ carboxylation processes at dawn and late in the photoperiod (Fig. 2; Griffiths et al., 1990;
Borland et al., 1993; Roberts et al., 1997). As demonstrated for Tillandsia utriculata in Phase I of CAM, low $C_4$-like discrimination accompanies PEPC activity as malic acid accumulates in the vacuole overnight. Typical for bromeliads, Phase II of CAM is rather brief, with a slight increase in net $CO_2$ uptake as the Rubisco discrimination signal begins to dominate carboxylation as PEPC is deactivated. During Phase III, stomata can be closed by high concentrations of $CO_2$ generated internally. The high levels of discrimination measured at this time are associated with $^{13}CO_2$ preferentially leaking from the leaf, reflecting the high inherent discrimination against $^{13}CO_2$ which is expressed by Rubisco. Under optimal conditions of water supply and PFD, stomata often re-open to allow carboxylation mediated directly by Rubisco (Phase IV), with PEPC becoming active again before dusk associated with the shift from $C_3$ to $C_4$ discrimination signal (Fig. 2). Additionally, over a 24 h period, analysis of the $^{13}C$ signal in metabolite pools allows the construction of detailed carbon budgets and provides an assessment of the relative contributions from $C_3$ and $C_4$ carboxylation to plant carbon balance (Borland et al., 1994; Borland, 1996).

There are difficulties in quantifying absolute rates of gas exchange (whether as $CO_2$ or $O_2$) or carboxylation (as the interplay between PEPC and Rubisco), without recourse to more complicated stable isotope or radiolabeling methodologies (Maxwell et al., 1998). Moreover, as outlined below, a number of ecological dogmas associated with CAM still need to be re-evaluated. Are higher-energetic costs, and large pools of storage carbohydrates, really limiting for growth in a high light environment? Are CAM plants really tolerant of high light and temperatures when morphological adaptations such as self-shading and leaf/cladode orientation, suggest that adaptations to reduce PFD interception are paramount in the most exposed habitats? Why, when similar life-forms of $C_3$ and CAM co-specifics are compared, do $C_3$ plants tend to predominate in the most exposed habitats, such as the outer crown of rainforest canopy or true desert regions such as Death Valley? Thus, are CAM plants truly xerophytes, when the possession of a suite of xeromorphic adaptations prevent substantial deviations in cellular water relations? CAM has evolved in so many families in response to contrasting selective pressures, including $H_2O$ limitation in the terrestrial habitat and $CO_2$ limitation in the aquatic habitat. Do the extremes of CAM distribution, from high rainfall/montane rainforests, to low temperatures...
in the Rockies represent relics (exceptions), or examples of how the metabolic plasticity central to CAM allows CO₂ uptake to be optimized over a 24 h period in succulent leaves? Ultimately, the answers to these questions lie in the metabolic and molecular regulatory mechanisms underpinning CAM. Here we have tissues which can tolerate a shift in internal conditions, whereby over a few hours, Rubisco may be operating under a range of CO₂ and O₂ concentrations equivalent to those encountered across palaeohistorical timescales. Our review in this chapter is framed by setting these conventional notions against recent developments using field and laboratory based approaches.

II. H₂O: Cellular Limitations Imposed by Deficit and Excess

A. Succulence

One of the diagnostic pre-requisites for CAM is succulence, whether at cellular or organ level: large chlorenchymatous, vacuolate cells comprise a homogenous population in many leaf succulents, typically in the Crassulaceae (Fig. 3) and Orchidaceae. Alternatively, the chlorenchyma may be heterogeneous, lying as a thin surficial layer surrounding photosynthetic stems or cladodes (e.g. the Cactaceae) or leaves (Agavaceae) above a large volume of water storage parenchyma (WSP). Many tropical epiphytes tend to show more complex arrays, with layers of WSP at the leaf surface in the Clusiaceae, (Borland et al., 1998) as well as being distributed throughout the leaf in the Bromeliaceae (Maxwell et al., 1994). Indeed, the Piperaceae may show more extreme differentiation with a multiple epidermis, palisade and spongy mesophylls showing variable carboxylation patterns (Nishio and Ting, 1988; Ting et al., 1994). Although WSP does not participate in the CAM cycle directly the contribution to water storage and recharge of chlorenchyma is important in the maintenance of overall tissue water status (Smith et al., 1987; Schulte et al., 1989; Yakir et al., 1994). However, it is interesting to note that in comparing three species of Peperomia and three species of Clusia, the cross-sectional areas of WSP are inversely correlated with the capacity for CAM (Gibeaut and Thomson, 1989; Borland et al., 1998). It is possible that the large cells of the WSP in these genera may not be capable of resisting water loss to the environment under extreme conditions (Kaul, 1977). Thus, a thicker cuticle together with a lower surface area: mesophyll volume ratio may be more effective in reducing water loss under conditions of extreme exposure, as found in the constitutive CAM species of Clusia (Borland et al., 1998).

B. Water Relations

The succulent CAM tissues have provided model systems for evaluating cellular water relations (Smith et al., 1986, 1987; Schulte et al., 1989; Murphy and Smith, 1994, 1998). Sections of stem or cladode have required innovative solutions to allow sealing into equipment such as the pressure chamber (such as epoxy resins or cyano acrylate adhesive; Murphy and Smith, 1994), but techniques such as the pressure probe allow direct quantitation of turgor pressure and water exchange characteristics (Murphy and Smith, 1994, 1998). Cell-sap extracts are dominated by vacuolar contents and thus provide an effective proxy for the osmotic consequences of day-night changes in tissue solute concentration and can be easily measured by osmometry on sap extracted from tissue. At the tissue level, there are inverse patterns of leaf water potential (ψ), with xylem tension becoming greater during Phase I and Phase IV when stomata are open. As malic acid accumulates, concomitant changes in leaf-sap osmotic pressure (π) are observed, which could act as a means to drive water uptake from dew fall at dawn (Smith et al.,

Fig. 3. Scanning electron microscope view of the closely packed mesophyll cells of Kalanchoe daigremontiana showing the lack of intercellular air space and scant cover of chloroplasts (from Maxwell et al., 1997).
Concomitant changes in turgor pressure ($P$) can be calculated from the difference between $\psi$ and $\pi$, but it has taken a careful study comparing indirect methods for analyzing $\psi$ and $\pi$, with direct measurements of $P$ (with the pressure probe) to show that the xylem osmotic component should be corrected when deriving $\psi$ leaf using the pressure chamber (Murphy and Smith, 1994). Most recently, the pressure probe has been used to validate a model of cell-water relations parameters, including fluxes between cells via plasmodesmata (Murphy and Smith, 1998).

At the ecological level, water potentials lower than $-1.0$ MPa are rarely encountered in CAM plants. For Aechmea aquilega, normally an epiphyte but growing terrestrially at a coastal site in Trinidad, a maximum xylem tension of 0.85 MPa was measured, compared to 3.3 MPa for a nearby $C_3$ shrub (i.e. equivalent to $\psi$ of $-3.3$ MPa). The dawn to dusk changes in $\psi$ during the onset of the dry season in Trinidad are shown in Fig. 4 for the hemiepiphytic strangler Clusia minor. It is notable that greater xylem tensions (more negative $\psi$) developed during the rainy season, when daytime gas exchange would have predominated. High levels of CAM activity were induced at the onset of the dry season (Borland et al., 1996), when rainfall ceased (Fig. 4a) and it is notable that the dusk values of xylem tension were reduced ($\psi$ more positive) at this time (Fig. 4b), indicating the effectiveness of CAM at conserving water on a daily basis.

These observations lead us to address two dogmas associated with CAM: one is that CAM provides higher water use efficiency than $C_3$ plants. When measured directly in terms of water use per unit increment of dry matter gain, or instantaneously during gas exchange, there is no doubt that $C_3$ mesophytes have lower WUE than CAM plants. However, when sympatric species with similar life-forms are compared under comparable environmental stress, WUE for $C_3$ and CAM are indeed similar (Griffiths et al., 1986; Eller and Farrari, 1997). Secondly, since CAM plants never develop severe water deficits, can we really describe them as tolerant of water deficits *sensu* true xerophytes? Succulent plants undoubtedly show remarkable adaptations to conserve water status and maintain carboxylation conductance in the face of severe diffusion limitation, as discussed below.

**C. Root Systems and Salinity**

The work of Park Nobel and colleagues has made a major contribution to our understanding of how the root/soil interface can be used to promote water uptake or prevent water loss in CAM spp, depending on soil water status (Jordan and Nobel, 1984; Nobel, 1992; North and Nobel, 1998). Mature roots, by shrinking and suberising in response to soil water deficits, create disjunctions in the soil and root hydraulic conductance pathway (Nobel, 1992), and abscission of lateral roots both reduce water uptake (or loss from the plant: Nobel, 1992; North and Nobel, 1998). Root growth is rapidly re-established within one week of re-wetting, driven by water supplied from the stem, allowing stem and leaf water storage capacity to be recharged rapidly even after extended drought periods (Nobel and North, 1998).

However, in contrast to water deficits, most CAM plants are not salt tolerant, with lateral root extension reduced by salt (Gersani et al., 1993). A comparison of stem succulent cacti and the true halophytic CAM plant *Mesembryanthemum crystallinum* (Lüttge, 1986) or remobilize water from older leaves (Ruess and Eller, 1985).
Anne M. Borland, Kate Maxwell and Howard Griffiths

1994) showed that when cacti are subjected to saline or hypersaline environments, necrosis and death in the seedling or juvenile stages of growth is common. The exception is the C₃-CAM intermediate M. crystallinum, in which responses to salinity and induction of CAM are definable in molecular terms (Adams et al., 1998; Cushman and Bohnert, this volume). Here, an initial suite of juvenile leaves (which can induce CAM: A. N. Dodd, personal communication) provide a critical mass of plant material using winter rains in the natural environment. As the soil dries, and salinity increases, there is a developmental shift to CAM which can be accelerated by appropriate environmental conditions (Adams et al., 1998).

D. Adaptations to Extreme Habitats: Excess Water

Given that CAM has evolved in so many families, and provides a model for analysis of the molecular and biochemical basis of responses to environmental stress, how can we account for the occurrence of CAM when water is not limiting? Aquatic macrophytes of the Isoetid lifeform, such as Isoetes lacustris and Littorella uniflora, develop CAM in the aquatic habitat and use CO₂ supplied from sediments to overcome the diffusive limitations imposed by the aquatic milieu, where CO₂ diffuses 10,000 x slower than in air. On exposure, the amphibious L. uniflora loses CAM activity, increasing investment in Rubisco while still relying on sedimentary CO₂ supplies (Robe and Griffiths, 1998; W. E. Robe, personal communication). This illustrates how CAM serves as an example of biochemical convergence in evolutionary terms, as a solution to diffusive limitation whether imposed externally (by water) or internally (by succulent leaf tissues: see below).

In the latter case, we should try to explain the distribution of CAM plants in extreme habitats. For instance, Opuntia humifusa can tolerate low temperature, high moisture habitats in Canada and eastern USA (Goldstein and Nobel, 1994; Section C below). Alternatively, endemic CAM bromeliads, such as Aechmea aripensis in Trinidad and A. laesseri in Venezuela, are often found exclusively in upper montane rainforest formations, in shaded environments where rainfall may exceed 5 meters y⁻¹! Is CAM in these habitats a relict of a previous drier environment, or could it be that CAM provides a competitive advantage over similar sympatric C₃-lifeforms? The photosynthetic plasticity which underpins the 4 phases of CAM could be the key for optimizing CO₂ gain where films of water on the surface of wetted leaves preclude CO₂ exchange for extended periods.

III. CAM as a Carbon Concentrating Mechanism: Morphological and Biochemical Considerations

A. Mesophyll Conductance and Photorespiration

A carbon concentrating mechanism (CCM) functions to generate an elevated partial pressure of CO₂ at Rubisco active sites (Pᵢ) and thereby increases carboxylation efficiency through a reduction in oxygenase activity. In considering CAM as a CCM, the importance of organic acid decarboxylation as a means of suppressing photorespiration has perhaps been exaggerated. Refixation of CO₂ during Phase III occurs at an internal CO₂ partial pressure (pCO₂) which may be as high as 0.8 to 2.5% (Spalding et al., 1979; Cockburn et al., 1979) and thus qualifies for CCM status. However, substantial Rubisco-mediated atmospheric CO₂ fixation occurs in many CAM and C₃-CAM plants during Phases II and IV (Borland and Griffiths, 1996). This component of day-time fixation is subject to the biochemical and biophysical limitations to carbon gain typical of C₃ plants (von Caemmerer and Farquhar, 1981; Farquhar and von Caemmerer, 1982) and does not constitute a functional CCM (Osmond et al., 1999).

CAM leaves are primarily designed for water conservation, requiring a leaf morphology which is strikingly at odds with the thin, porous blade that is optimal for atmospheric CO₂ fixation (Maxwell et al., 1997; Osmond et al., 1999). CAM plants tend towards thick, succulent leaves (Teeri et al., 1981; Winter et al., 1983; Osmond et al., 1999) or photosynthetic stems. The large, highly vacuolate cells are tightly packed creating minimal volumes of air space (Smith and Heuer, 1983). In leaves of Kalanchoë daigremontiana the highly succulent nature of the cells mean that air space comprises only 8% of leaf volume (Maxwell et al., 1997).

Since CO₂ and O₂ compete for Rubisco active sites, the efficiency and capacity of C₃ photosynthesis is strongly influenced by Pₑ. Pₑ is influenced by the internal (mesophyll) conductance of CO₂ (gₑ) from
stomatal cavity to Rubisco. Recently, a positive correlation has been established between $g_e$ and intracellular air space and a negative relationship between $g_e$ and leaf thickness (Evans and von Caemmerer, 1996; Chapter 14, Evans and Loreto). Although a high diffusive resistance to CO$_2$ had previously been postulated for succulent leaves (Robinson et al., 1993b), it was calculated that in leaves of *K. daigremontiana* during Phase IV, $g_e$ was 0.05 mol CO$_2$ m$^{-2}$ s$^{-1}$ bar$^{-1}$, manifested as a value of $P_e$ of only 10$^9$ μbar at an ambient pCO$_2$ of 350 μbar (Maxwell et al., 1997). For comparison, a $P_e$ of 209 μbar was calculated for tobacco leaves which contained a similar amount of Rubisco (Evans et al., 1994). Assuming an internal concentration of 21% O$_2$ and 0.01% CO$_2$, a ratio of carboxylation to oxygenation will be close to parity, as compared to 2.9 for spinach (Osmond et al., 1999). Comparably low values of $g_e$ have been demonstrated in other CAM species, most notably for three *Clusia* species which exhibit a gradation of CAM activity (C$_3$, C$_4$-CAM and obligate CAM) which correlated with both succulence and decreasing $g_e$ (Borland et al., 1998; Gillon et al., 1998). Such exceptionally low values of $g_e$ limit photosynthetic capacity of succulent species during Rubisco-mediated atmospheric CO$_2$ uptake. Moreover, while conventional estimates of $P_e$ (the partial pressure of CO$_2$ within the sub-stomatal cavity) approximate $P_e$ in a number of thin-leafed species, in succulent leaves $P_e$ may be up to three-fold higher than the actual partial pressure of CO$_2$ which is presented to Rubisco in the chloroplast (Maxwell et al., 1997).

Light-dependent $^{18}$O$_2$ uptake of intact leaf discs can be used to investigate the nature of O$_2$ uptake during CAM (Thomas and André, 1987). Figure 5 shows CO$_2$ assimilation concomitant with O$_2$ uptake (corrected for dark respiration) as a function of ambient [CO$_2$] in *Dendrobium speciosum*. During Phase IV, a low stomatal and internal conductance to CO$_2$ assure low rates of CO$_2$ assimilation and a clear negative relationship is observed between ambient CO$_2$ concentration and O$_2$ uptake (Fig. 5a). In this instance, photorespiration comprised 80% of electron transport at 590 μbar CO$_2$ (Maxwell et al., 1998).

During decarboxylation, being a thick leaf has mixed blessings. Firstly, high diffusive limitation coupled to stomatal closure prevents excessive CO$_2$ leakiness during decarboxylation. However, elevated intracellular pO$_2$ is generated from linear electron transport (approximately 42%: Spalding et al., 1979), which at the very least increases the possibility of photorespiration during the CCM of Phase III (Maxwell et al., 1998), but, equally, may promote the formation of potentially damaging reactive oxygen species (Osmond et al., 1999). Indeed, increases in the transcription of genes which encode scavenging enzymes have been observed during the induction of CAM in *M. crystallinum* (Miszalski et al., 1998). The activities of ascorbate peroxidase and superoxide dismutase have also been shown to increase with the shift to CAM in *Sedum album* (Castillo, 1996). During Phase III, high rates of O$_2$ uptake have been observed (Thomas and André, 1987; Cote et al., 1989; Maxwell et al., 1998) which, given the biochemical and kinetic properties of Rubisco, seem only partially attributable to photorespiration (Osmond et al., 1999). In the example provided in Fig. 5b, O$_2$ uptake was high over the entire CO$_2$ range (1.2 μmol m$^{-2}$ s$^{-1}$ at 1.4 % CO$_2$) and showed only a slight CO$_2$ sensitivity. It is possible that during Phase III, a significant proportion of electron transport is used to reduce O$_2$ via the Mehler ascorbate...
peroxidase pathway, especially given the highly oxidizing conditions which prevail during Phase III (Osmond et al., 1999).

Accepted dogma states that CAM is a carbon concentrating mechanism and thus photorespiration should be a negligible metabolic process. However, the morphological constraints of the succulent leaf required to support CAM result in low partial pressures of CO₂ within the leaf, which increase the probability of photorespiration throughout the day. It is clear that photorespiration occurs in CAM plants, often at very substantial rates and must be significant in CAM-idling plants.

B. PEPC Regulation

The day/night separation of metabolism in CAM requires that PEPC is effectively down-regulated at the start of the day during Phase II. Although Phase II is usually considered as a relatively brief transition between C₄ and C₃ carboxylation processes, a literature survey of a range of obligate and facultative CAM species has indicated that up to 50% of CO₂ taken up over 24 h can occur during Phase II (Borland and Griffiths, 1996). It seems likely that an appreciable amount of this morning uptake is mediated by PEPC. Field and laboratory-based studies on hemi-epiphytic stranglers of the genus Clusia have provided direct evidence that PEPC can remain active for 4–5 h after dawn as indicated by: i) a continued accumulation of organic acids over this period and ii) the low values of instantaneous carbon isotope discrimination measured during leaf gas exchange (Borland et al., 1993; Roberts et al., 1997). Flux through PEPC is regulated by reversible phosphorylation which reduces the sensitivity of the enzyme to inhibition by L-malate, with the phosphorylated, malate-insensitive (active) form of PEPC usually assumed to be present only at night (Nimmo et al., 1984; 1986; Carter et al., 1996). In C. minor however, PEPC can remain phosphorylated for some 3 h into the photoperiod, as indicated by measurements of the sensitivity of the enzyme to malate inhibition in vitro (Fig. 6b), while in K. daigremontiana, the enzyme is rapidly de-phosphorylated within the first hour of the photoperiod (Fig. 6a; Borland and Griffiths, 1997). Similar investigations on Tillandsia usneoides, an extreme atmospheric bromeliad, indicate that PEPC is only slowly de-phosphorylated some 5 h into the photoperiod (R. Haslam and A. M. Borland, unpublished). Such observations call for a reconsideration of the extent of day-time PEPC activity in diverse CAM species.

Previously, reports have suggested that the amount

![Fig. 6. Apparent activation of PEPC (a,b) and Rubisco (c,d) measured in vitro for extracts prepared from leaves of Kalanchoë daigremontiana and Clusia minor. Diel changes in Kᵢ malate (a,b) indicate the apparent phosphorylation status of PEPC in control leaves and in leaves prevented from accumulating malate overnight in an atmosphere of N₂ but transferred to ambient air at the start of the photoperiod (Borland and Griffiths, 1997). The activation status of Rubisco (c,d) is indicated by changes in initial and final (pre-incubation with Mg²⁺) activities over the photoperiod (A. Roberts and H. Griffiths, unpublished). The solid bars on the x-axes indicate the periods of darkness.]
of \( \text{CO}_2 \) taken up during Phase II may be influenced by the magnitude of \( \text{CO}_2 \) fixation and malic-acid accumulation during the previous night (Medina and Delgado, 1976; Fischer and Kluge, 1984). Physiological manipulations of leaf malate content and perturbation of malate compartmentation between vacuole and cytosol have suggested that the circadian rhythm of PEPC phosphorylation (Nimmo et al., 1987) may be modulated by malate (Borland and Griffiths, 1997; Grams et al., 1997a). As illustrated in Fig. 6a,b, if leaves of \textit{C. minor} or \textit{K. daigremontiana} are prevented from accumulating malate overnight in an atmosphere of \( \text{N}_2 \), subsequent transfer to ambient air at the start of the photoperiod results in an increase in apparent phosphorylation status of PEPC for the first 2–3 h of the photoperiod and de-phosphorylation now occurs some 3–4 h into the light in both species (Borland and Griffiths, 1997). The enhanced phosphorylation status of PEPC is accompanied by an increase in net \( \text{CO}_2 \) uptake during Phase II in leaves prevented from accumulating malate (Borland and Griffiths, 1997). Thus, the degree of PEPC phosphorylation can modulate carbon gain in response to short-term changes in the environment which alter the amount and/or partitioning of malate between vacuole and cytosol.

Maintenance of PEPC activity for much of the day implies that futile cycling of malate synthesis/decarboxylation could represent a substantial proportion of day-time carbon flux. A variety of experimental approaches have illustrated that futile cycling can and does occur during Phase IV in a range of CAM species (Osmond et al., 1996). However during Phase II in \textit{C. minor} estimates of PEPC activity in vivo (obtained from instantaneous carbon-isotope discrimination), compared closely with the amount of malate accumulated over this period, implying that decarboxylation and thus futile cycling, were curtailed (Borland and Griffiths, 1997).

\section*{C. Rubisco Regulation}

The capacity for optimizing carbon gain and WUE by variably adjusting the amount of \( \text{CO}_2 \) fixed during the 24 h CAM cycle also requires a consideration of Rubisco regulation, particularly since it is the direct uptake of \( \text{CO}_2 \) via \( \text{C}_3 \) carboxylation during Phase IV which largely determines the growth and productivity of CAM species (Nobel, 1996). Studies conducted on \( \text{C}_4 \) plants have shown that Rubisco activity may be modulated in response to changes in light intensity, \( \text{CO}_2 \) or \( \text{O}_2 \) supply through reversible carbamylation, facilitated by the action of Rubisco activase (Portis, 1995). This aids the binding of \( \text{Mg}^{2+} \) to form a catalytically active complex. Other mechanisms of control include the binding of naturally occurring inhibitors such as 2-carboxyarabinitol 1-phosphate (CA1P) and modulation by stromal metabolites (Seemann et al., 1990; Geiger and Servaites, 1994; Parry et al., 1997). The existence of this variety of regulatory mechanisms increases the responsiveness of Rubisco to changes in the environment, and thus should serve to modulate \( \text{C}_3 \) carboxylation to the fluctuations in \( \text{CO}_2 \) supply which occur over the daytime phases of CAM. Although the diurnal regulation of Rubisco has previously received little attention in CAM plants, recent investigations have indeed indicated that in \textit{K. daigremontiana} and \textit{C. fluminensis}, both initial and final Rubisco activities measured in vitro change over the course of the day (Fig. 6c,d; A. Roberts and H. Griffiths, unpublished). In both species, the activity and the percentage activation of Rubisco, which reflects carbamylation state, is highest towards the end of the day when decarboxylation is complete, stomata have re-opened and net \( \text{CO}_2 \) uptake is evident. The up-regulation of Rubisco at this time should serve to maintain carboxylation strength (and WUE), thereby compensating in some measure for the diffusive limitations to \( \text{CO}_2 \) encountered during Phase IV (Maxwell et al., 1997; 1998). However, it is likely that \( \text{CO}_2 \) supply may also be limiting during Phase II if PEPC remains active as discussed above. Since PEPC has a high substrate affinity (i.e. is a more efficient scavenger of \( \text{CO}_2 \) than Rubisco), this will create an enormous ‘biochemical resistance’ to \( \text{CO}_2 \) in addition to that imposed by the closely packed succulent cells of the leaf. In this situation, the reduced catalytic activity of Rubisco during Phase II might reflect the inability of the chloroplast to generate RuBP.

The low activities of Rubisco measured during Phase II may also be attributed, at least in part, to the binding of endogenous inhibitors such as CA1P. The presence of such inhibitors, as indicated by the dark inactive state of Rubisco has been reported for a number of CAM species including \textit{Ananas comosus} and \textit{Crassula argentea} (Cu et al., 1984; Servaites et al., 1986). Moreover, studies on \( \text{C}_4 \) plants have indicated that CA1P binding is more prevalent in the morning than at an equivalent PFD later in the day (Sage et al., 1993). An additional component of Rubisco modulation may involve chloroplastic
metabolites. In C₃ plants, the concentrations of RuBP and PGA can change substantially and often reciprocally during the course of a day, producing dramatic and rapid changes in Rubisco activities (Portis, 1995). Although little is known about the intracellular distribution and diel changes of metabolites in CAM cells, it is known that levels of PGA in K. daigremontiana increase during deacidification and RuBP does not reach steady state for at least 20 min after onset of the photoperiod (Kenyon et al., 1981).

The extent to which Rubisco is regulated via inhibitors such as CA1P, versus changes in carboxylation state of the enzyme may well differ amongst CAM species, as has been shown to be the case in C₃ plants (Holbrook et al., 1994). Indeed, the unique conditions encountered during the day in CAM species suggest that hitherto unsuspected subtleties and complexities of Rubisco regulation may underpin the plasticity of day-time gas exchange patterns. Depending upon the CAM species, these can range from the continuous day-time net CO₂ uptake found in CAM cycling to the extreme condition of CAM-idling where stomata remain closed over 24 h.

**D. Diel Carbohydrate Partitioning**

A major metabolic constraint imposed by the CAM cycle is the requirement for a large reciprocating pool of carbohydrates which must be distinguished from the carbon skeletons destined for nitrogen metabolism, respiration and growth. During Phase III, 75% of the carbohydrates synthesized via gluconeogenesis and from the refixation and processing of CO₂ via C₃ photosynthesis, must be retained as a reserve to fuel carbon assimilation over the subsequent night. The remaining carbohydrates, together with any produced as a result of Phase IV photosynthesis are redirected towards growth. The biochemical processes which control this partitioning of carbohydrates are poorly understood but appear to be regulated in such a manner that the demands of the dark reactions of CAM take precedence over export. Evidence for this stems largely from source-sink manipulations of K. pinnata, where decreasing sink demand by girdling or removing young leaves resulted in the accumulation of sucrose and abolishment of Phase IV photosynthesis, while dark CO₂ uptake was unaffected (Mayoral et al., 1991). Moreover, increasing sink strength by darkening leaves did not affect CAM activity in the illuminated source leaf.

Understanding the metabolic processes which regulate carbohydrate partitioning in CAM plants is complicated by the diversity of strategies for cycling carbon into different carbohydrate pools. Two main groups of CAM species are commonly distinguished in terms of the most abundant storage carbohydrate; i) chloroplastic starch and glucan accumulators which include many members of the Crassulaceae such as K. daigremontiana, K. pinnata and Sedum telephium (Sutton, 1975; Fahrendorf et al., 1987; Borland, 1996), ii) extra-chloroplastic soluble sugar or polysaccharide accumulators which include Ananas comosus (vacuolar mono and di-saccharides; Carnal and Black, 1989), Fourcroya humboldiana (fructans; Olivares and Medina, 1990) and Aloe arborescens (polymer of galactose and mannose; Verbücheln and Steup, 1984). In addition, some CAM species, including several within the genus Clusia, accumulate both soluble sugars and starch (Popp et al., 1987; Borland et al., 1994). Such complexity is compounded by the recent observations of Christopher and Holtum (1996; 1998) who have defused the widely accepted view that decarboxylation biochemistry (malic enzyme or PCK) determines whether carbohydrates are stored inside or outside of the chloroplast. At the last count, at least eight patterns of carbohydrate partitioning in more than twenty CAM species have been distinguished.

One approach for examining the arrangement of carbon pools in CAM plants is via quantitative measurements of the carbon-isotope composition of the various organic fractions in the leaf. Since carbohydrates synthesized directly from C₃ photosynthesis are depleted in ¹³C compared to those arising from the day-time decarboxylation of malate, studies on K. blossfeldiana and K. daigremontiana have suggested that carbohydrates derived from C₃ and C₄ metabolism are channeled into parallel pathways of glycolysis at night (Deléens and Garnier-Dardard, 1977). Thus, respiratory CO₂ was generated from soluble sugars depleted in ¹³C whereas PEP was produced from the glycolytic breakdown of ¹³C-enriched starch (Deléens et al., 1979). In species such as C. minor where degradation of both soluble sugars and starch is necessary to supply PEP, it is likely that the situation will be more complex at night. However, there does appear to be compartmentation between the products of C₃ and C₄ carboxylation during the day in C. minor (Borland et
Under natural field conditions, carbon lost from soluble sugars at night was considerably enriched in $^{13}$C compared to CO$_2$ fixed during Phase IV, indicating the existence of two soluble sugar pools: i) a vacuolar pool enriched in $^{13}$C from the decarboxylation of organic acids and ii) a rapidly turned over cytosolic pool of transport sugars synthesized as photosynthetic end-products during Phase IV (Borland et al., 1994). Such compartmentation provides a means by which the metabolic pathways that constitute CAM and those that fuel growth could be regulated independently of one another.

IV. Daily Integration of Environmental Conditions

A. Modulation of the CAM Phases

CAM plants tend to succeed in extreme environments which are subject to daily fluctuations in precipitation, integrated/incident photon flux density (PFD) and temperature. The expression of CAM is thus characterized by a highly plastic response to environmental perturbations which permits survival under variable adverse conditions. Over the course of 24 h, the amplitude of each of the four phases of gas exchange is determined by environmental and physiological factors and is highly species dependent (Borland and Griffiths, 1996). Under extreme conditions of drought stress, stomata may remain closed over 24 h, behind which the refixation of respiratory CO$_2$ at night and decarboxylation of organic acids by day permits continued flux through the pathways of carbon metabolism and electron transport. Such CAM-idling is particularly well-documented for shallow-rooted cacti (Kluge and Ting, 1978). CAM-cycling represents a less extreme modification of CAM, where continuous uptake of CO$_2$ over the day is accompanied by nocturnal accumulation of acids derived from refixation of respiratory CO$_2$. This particular mode of photosynthetic metabolism is present in over 40 species representing at least 15 families (Martin et al., 1988), which are often found growing in habitats where water supply is unpredictable on a daily basis.

The degree of exposure is a strong determinant of C$_3$ and C$_4$ carboxylation during CAM. Figure 7 shows measurements of net CO$_2$ uptake made on the same day in the dry season in Trinidad on fully exposed and shaded C. minor plants. The shaded plants show much reduced rates of gas exchange during Phase II and particularly during Phase IV. Additionally the magnitude of CAM, measured as the dawn-dusk difference in titratable acidity ($\Delta$H$^+$) was 50% lower for the shaded plants. On-line carbon-isotope discrimination has illustrated the dominance of the C$_4$ discrimination signal during Phase II, indicating the magnitude of prolonged PEPC activity under field conditions (Borland et al., 1993; Roberts et al., 1997).

Incident and integrated PFD also has important implications for the degree of nocturnal CO$_2$ uptake with a direct relationship between the amount of organic acids accumulated overnight and the photon receipt of the previous photoperiod (Nobel and Hartsock, 1983 and Fig. 8). This relationship can be attributed to the increased accumulation of glucans under high PFD which are subsequently used to generate PEP, the carbon substrate for C$_4$ carboxylation at night. In a study of four cactus species, Nobel (1988) demonstrated that the degree of nocturnal acidification exhibited an initial linear increase with daily photon dose and saturated at around 30 mol photon m$^{-2}$ d$^{-1}$, presumably when vacuolar capacity was reached (Fig. 8; Nobel, 1988). A minimum of 3 mol photon m$^{-2}$ d$^{-1}$ was required for acidification, equivalent to an incident PFD of 69 $\mu$mol photon m$^{-2}$ s$^{-1}$ over a 12 h photoperiod. A similar pattern was observed for Agaves, although in contrast, a strikingly different pattern was obtained...
with the facultative CAM species *C. minor* (Fig. 8). Little evidence of light saturation of ΔH⁺ is observed, in this species which may accumulate over 1.5 M H⁺ during the dry season in Trinidad (Fig. 8; Borland et al., 1992). *Clusia* species accumulate both malate and citrate with an increasing contribution from citrate at higher PFD (Borland et al., 1996). Initial observations suggested that the degree of nocturnal acidification was correlated with the level of dusk acidity (Zotz and Winter, 1993b). This view has been modified since it has now been shown that dusk citrate concentration is negatively correlated with the amplitude of nocturnal fixation (Borland et al., 1996; Roberts et al., 1998). It has been suggested that in certain CAM species, the decarboxylation of citrate in high light generates additional carbon skeletons required for increased PEPC activity and thus modulates the expression of CAM in response to environmental conditions (Borland et al., 1996).

### B. Sun and Shade Distribution

CAM plants tend to colonize extreme habitats (Lüttge, 1987) and are likely to be either fully exposed or only partially shaded. This skewed distribution towards higher light environments may be explained by the increased energetic requirements of the regeneration of PEP during Phase III photosynthesis as compared to C₃ carboxylation (Winter and Smith, 1996) and the related minimum photon dose requirement for nocturnal acidification (Nobel, 1988). For example, the epiphytic bromeliad flora of Trinidad, W. Indies has been characterized according to habitat preference within the forest canopy viz. exposure, sun and shade tolerant (Pittendrigh, 1948). All CAM species are confined to the middle to top of the forest canopy (Griffiths and Smith, 1983). Indeed, relatively few CAM plants are distributed in deep shade, although notable exceptions exist which prove this rule. For example, *Aechmea magdalenae* is a terrestrial bromeliad which successfully colonizes both deep shade and exposed habitats (Skillman and Winter, 1997). This species has overcome the problem of light limitation through an effective and rapid utilization of sunflecks which may be sufficient to support CAM within the forest understory (Skillman and Winter, 1997).

The majority of CAM plants are able to adjust the photosynthetic apparatus to reduced light regimes, yielding responses which are typical of C₃ and C₄ plants (Adams et al., 1987), albeit at the expense of reduced nocturnal acidification (Nobel and Hartsock, 1983, Nobel, 1988). Herppich and co-workers (1998a,b) have recently made a thorough examination of photosynthetic acclimation to high and low light in the CAM-cycling plant, Delosperma tradescantoides, which grows under exposed and semi-shaded conditions. The shaded plants exhibited a 50% reduction in diurnal CO₂ assimilation, lower dark respiration rates and light compensation point, while the quantum yield of photosynthesis was comparable with high light plants, i.e. a set of responses which are identical to those predicted for C₃ plants.

### C. Photoinhibition and Photoprotective Strategies

In a consideration of the selective advantages of the evolution of CCMs, it has been postulated that CAM may prove effective in the alleviation of photoinhibition (Gil, 1986; Griffiths, 1989), as defined by a long-term decrease in photochemical efficiency, which may incorporate damage to the photochemical apparatus. In any photosynthetic organism, photoinhibition will arise when absorbed light energy exceeds the capacity for photosynthetic light use and thermal dissipation of the excess, parameters which are genotypically and/or phenotypically fixed. In all cases, acclimation to high light facilitates photoprotective...
tection and sun plants will tolerate a higher threshold PFD than shade plants, even under circumstances when shade plants exhibit CAM (Adams et al., 1987; Adams and Osmond, 1988).

The first line of defense against photoinhibition is a physical reduction in the amount of light incident on the light harvesting apparatus. CAM leaves tend towards a high reflectance of incident light, possibly as a consequence of water storage tissues and a thick epidermis. Laminar reflectance was 21% of incident light in *Hoya carnosa*, 40% in highly exposed leaves of *Dendrobium speciosum* (K. Maxwell, unpublished) and approximately 36% in young leaves of *Ananas comosus* (Adams et al., 1986). Development of wax in young leaves of *Cotyledon orbiculata* was strongly correlated with an increase in light reflectance (57% at day 35: Barker et al., 1997). Removal of wax from the leaf surface resulted in chronic and extremely prolonged photoinhibition in this species providing unequivocal evidence that wax constitutes a highly effective photoprotective barrier (Robinson et al., 1993a). Additionally, it has been suggested that anthocyanins may mitigate photoinhibition in young leaves of *C. orbiculata* prior to wax deposition (Barker et al., 1997).

In CAM species, the predicted phases of the pathway impose a contrasting pattern of photosynthetic light use over the diurnal course which may mediate photoprotection and photoinhibition. It has long been postulated that since decarboxylation events are energetically costly and are coincident with the periods of maximum incident PFD, photoinhibition will be minimized during Phase III. Indeed, a very large body of evidence has emerged in support of this hypothesis and it seems clear that decarboxylation will alleviate photoinhibition (Osmond, 1982; Adams et al., 1988; Griffiths et al., 1989) provided that the plants are pre-acclimated to high light (Adams et al., 1987; Adams and Osmond, 1988; Maxwell et al., 1994; Grams et al., 1997b; Roberts et al., 1997; Barker et al., 1998; Herppich et al., 1998a,b). The overnight accumulation of citrate has been observed to increase in certain plants exposed to high light and it has been suggested that decarboxylation of this organic acid may generate a higher pCO₂ compared to malate, and thereby prevent photoinhibition (Haag-Kerwer et al., 1992; Grams et al., 1997b), although a precise photoprotective role has yet to be substantiated. Phase II occurs at the beginning of the photoperiod when light levels are relatively low and therefore the likelihood of photoinhibition is reduced, although this will in part depend upon leaf orientation (Barker and Adams, 1997; Barker et al., 1998). Extended PEPC activity may delay decarboxylation until PFD is maximal and thereby maximize photosynthetic light use when the risk of photodamage is greatest (Roberts et al., 1998).

The duration of Phase III decreases with increasing PFD and subsequent Phase IV activity represents Rubisco mediated atmospheric CO₂ uptake. Despite very low internal pCO₂, this phase is coincident with high Rubisco activity (Fig. 6c,d). Since photosynthesis rates are high at this time (Maxwell et al., 1997, 1998), it is feasible that Rubisco oxygenase maintains photon use and alleviates photoinhibition in CAM plants in a mechanism identical to that found in C₃ plants (Heber et al., 1996; Osmond et al., 1997).

At light levels which exceed photosynthetic saturation, a proportion of absorbed light energy may be harmlessly dissipated from the light harvesting complex, in a photoprotective process which is manifested as an increase in non-photochemical quenching (NPQ) of chlorophyll fluorescence (Horton et al., 1996). Almost without exception, dissipation is linked with the de-epoxidation of the carotenoids violaxanthin to antheraxanthin and zeaxanthin within the light harvesting complex, in reactions which comprise the xanthophyll cycle (Demmig-Adams and Adams, 1996; Horton et al., 1996). Daily variations in both the expression of NPQ and the xanthophyll cycle have been observed in CAM species.

On a daily basis, the highest values of NPQ (Winter and Demmig, 1987) and zeaxanthin (Winter et al., 1990) have been observed during the transient phases of CAM. These observations, made at constant light intensity, reinforce the concept of an increased requirement for thermal dissipation when light use is lowest. However, despite high levels of titratable acidity and delayed decarboxylation, significant rates of thermal dissipation are also required to prevent chronic photoinhibition during Phase III in the genus *Clusia* (de Mattos et al., 1997; Grams et al., 1997b; Roberts et al., 1998). The operation of the xanthophyll cycle during CAM has most elegantly been described in recent work on two *Opuntia* species (Barker and Adams, 1997; Barker et al., 1998). Both exhibit a positive relationship between photon dose and the xanthophyll pool size in cladodes with different orientations. Equally, both studies provide convincing evidence for a dependency of thermal dissipation on the formation of antheraxanthin and zeaxanthin.
Cladodes of *Opuntia stricta* are subject to sub-zero winter temperatures during the early morning and are thought to decrease photon use by an inhibition of Calvin cycle enzymes and a reduction in membrane fluidity which prevents malic acid efflux and decarboxylation (Barker et al., 1998). Therefore, when low temperatures coincide with high incident PFD, the increased probability of photoinhibition is clear. Under these conditions, the cladodes exhibited significant reductions in photochemical efficiency, which was linked to xanthophyll cycle activity and whereby approximately 80% absorbed energy was diverted through energy dissipation (Barker et al., 1998). North-facing cladodes retained a high proportion of de-epoxidized xanthophylls overnight, thereby effectively creating a system which is poised for thermal dissipation at the onset of adverse conditions the following morning (Maxwell et al., 1995). With increasing temperature, a greater proportion of light energy was used photochemically, coincident with decarboxylation events (Barker et al., 1998). Very similar responses have been observed with cladodes of *O. macrorhiza* growing in North America (Barker and Adams, 1997) and are likely to be typical for a number of CAM species subject to combined light and chill stresses.

Epiphytes are subject to large diurnal and seasonal variations in the light environment which necessitate key, rapid adjustments in the photosynthetic apparatus, despite extreme resource limitation. Mature leaves may experience a 60% increase in integrated PFD within 5–6 days and an extreme response is predicted if species are to succeed within the epiphytic niche (Maxwell et al., 1992, 1994, 1995). Figure 9 provides a summary of the photoprotective strategies employed by the C₄-CAM epiphytic bromeliad *Guzmania monostachia* over a diurnal course during the dry season in Trinidad, 1995. Net CO₂ assimilation rates are characteristically low and photon use during the middle of the day is supplemented by the reversible induction of CAM (Fig. 9b). Despite high photorespiratory activity, photon use still only accounted for 10% absorbed light energy. With such low photosynthetic activity, photoinhibition is predicted, although PS II was maintained in an oxidized state, manifested as a high level of photochemical quenching (qP = 0.596 at 12:30). Photoprotection was mediated by exceptionally high levels of thermal dissipation coupled to zeaxanthin formation (Fig. 9d).

In summary, effective photoprotection requires a regulated interplay between absorbed light energy use and dissipation, particularly in CAM plants, whereby CO₂ supply, pCO₂ and Rubisco activity varies considerably both over the diurnal course, imposed by the photosynthetic pathway and in response to environmental variables.

V. Seasonal Integration of CAM Performance and Productivity

A. Seasonal Changes in Constitutive CAM Expression

The dynamic shifts in carboxylation processes described above serve to illustrate the range of biochemical and molecular mechanisms which fine-tune carbon gain to fluctuations in the environment. However, it is important that laboratory-based manipulations are viewed in the ecophysiological context by considering seasonal and longer-term changes in the expression of CAM. Water is the key environmental variable limiting CO₂ uptake by desert
succulents and long-term water use efficiencies integrated over an entire year can range from 0.016 \( \text{CO}_2/\text{H}_2\text{O} \) in *Agave deserti* to 0.005 \( \text{CO}_2/\text{H}_2\text{O} \) in *Opuntia basilaris* (Szarek and Ting, 1974; Nobel, 1976). Despite the seasonal and annual variations in precipitation in many of the world's deserts, under natural conditions values of carbon isotope discrimination (\( \Delta \)) for stem succulents such as agaves and cacti are remarkably conserved, ranging from only 2–10 %o (Griffiths, 1992). Such observations may be related to low rates of \( \text{CO}_2 \) uptake during Phases II and IV (in comparison to leaf succulents), which may be abolished under severe drought. Under more mesic conditions, the proportion of day-time \( \text{CO}_2 \) uptake may increase in stem succulents, but any effect on \( \Delta \) has been shown to be masked by a concomitant increase in dark \( \text{CO}_2 \) fixation (Griffiths, 1992). Some desert succulents can experience severe drought when successive years without rainfall lead to CAM-idling. Such long-term responses are possible because of the remarkable tissue tolerance to water loss in cacti and agaves. Some species can lose 80–90% of their water content and still survive, sometimes up to six years without water in the case of the cactus *Copiapoa cinerea* (Nobel, 1988). In such situations, the cycling of carbon between the \( \text{C}_3 \) and \( \text{C}_4 \) pathways behind closed stomata serves to maintain photosynthetic integrity and a positive carbon balance at the expense of growth.

Aside from deserts, the other major biome for CAM species is the tropical rainforest, which tends to conjure an image of relatively stable environmental conditions. However, many seasonal forest formations are subject to increasing drought and light intensity as the dry season progresses, with concomitant changes in temperature and vapor pressure deficit. This combination of ‘stresses’ can enhance the expression of CAM in constitutive CAM species; the extreme atmospheric bromeliad *T. usneoides* increases investment of leaf proteins in PEPC and PCK in response to long-term drought/high light (R. Haslam, H. Griffiths and A. M. Borland, unpublished). In many leaf succulents of the genus *Kalanchoë*, rather than switching to CAM-idling in conditions of severe drought, dark \( \text{CO}_2 \) uptake is maintained since leaf water status can be preserved through the mobilization of water reserves from the abscission of older leaves (Kluge and Brulfert, 1996).

### B. Seasonal Carbon Gain in Facultative CAM Plants

Facultative CAM comprises both annual and perennial species which tend to use the \( \text{C}_3 \) pathway to establish high rates of growth at times of sufficient water supply but switch to CAM as a means of reducing water loss while maintaining photosynthetic integrity during dry periods. Measurements of carbon-isotope discrimination (\( \Delta \)) in leaf organic material are commonly used to identify and quantify the contribution of CAM to seasonal carbon gain within plant populations (Griffiths, 1992). The unprecedented range of CAM expression which can be provoked by environmental manipulations for different *Clusia* species for example is generally reflected in leaf \( \Delta \) taken from plants growing in their natural habitats (Popp et al., 1987; Franco et al., 1994). Thus \( \Delta \) can range from 6‰ in *C. rosea* growing in dry seasonal forest where \( \text{CO}_2 \) uptake by PEPC dominates photosynthetic carbon metabolism to 20‰ in *C. aripoensis* growing in moist montane rainforest where most \( \text{CO}_2 \) uptake is taken up by Rubisco over the annual cycle of leaf growth (Popp et al., 1987; Borland et al., 1992). However, organic \( \Delta \) is dominated by structural material which comprises at least 50% of leaf dry weight (Borland et al., 1994). Thus, if leaves flush during periods of adequate rainfall, the deposition of structural material will be dominated by a \( \text{C}_3 \) signal which may obscure the later contributions from \( \text{C}_4 \) carboxylation, which in the case of species such as *C. minor* (\( \Delta = 20\% \)) can be substantial (Borland et al., 1993).

A more direct assessment of the relative contributions of \( \text{C}_4 \) photosynthesis and CAM in a facultative CAM species is provided by the elegant studies of Zotz and Winter (1993b; 1996). Direct measurements of leaf gas exchange at intervals over an entire growing season demonstrated that CAM dominated carbon gain in epiphytic individuals of *C. uvitana*. In contrast, while dark \( \text{CO}_2 \) uptake contributed significantly to diel carbon gain in hemi-epiphytic plants on most days throughout the year, day-time photosynthesis usually exceeded night-time uptake. From this, the authors concluded that light was the main climatic factor determining carbon gain and expression of CAM in hemi-epiphytic plants while water availability was the critical factor in determining CAM expression in epiphytic plants (Zotz and Winter, 1996). These studies also revealed a close correlation
between maximum instantaneous rates of net CO₂ uptake and total 24 h carbon gain in *C. uvitana*, as has been found for C₃ plants (Zotz and Winter, 1993a). Extending this approach to a range of sympatric C₃ and CAM epiphytes growing in a moist semi-evergreen rainforest indicated that annual carbon gain and long-term nitrogen use efficiency were similar for all the species although long-term water use efficiency in the CAM species was more than twice as high as in the two C₃ epiphytes investigated (Zotz and Winter, 1994).

While the studies of Zotz and Winter would tend to reinforce theoretical considerations that the energetic costs associated with CAM are at most only 10% higher than those attributed to C₃ photosynthesis (Winter and Smith, 1996), there can be occasions when under extreme and unpredictable environmental conditions in nature, CAM might represent a liability in terms of plant carbon balance. Field investigations of a sympatric weak CAM and constitutive CAM species of *Clusia* growing on sand dune formations in Rio de Janeiro revealed that after a particularly prolonged dry season with record high temperatures, the CAM species became deciduous while the weak CAM species remained green (Roberts et al., 1996). This surprising observation might reflect the energetic costs and requirements for large pools of carbohydrates committed to the CAM pathway. More recently, the generation of constitutive CAM mutants of *M. crystallinum* in which the juvenile C₃ growth phase is abolished, suggest that the extremely slow growth rate of these mutants reflects the costs associated with the CAM pathway (Adams et al., 1998).

A variation of C₃-CAM intermediary is exhibited by plants showing CAM-cycling where diel fluctuations in malate are found but all uptake of external CO₂ occurs during the day. The rapid induction or enhancement of CAM-cycling serves to recapture increasing amounts of respiratory CO₂ at night under conditions of increasing drought (Borland and Griffiths, 1990, 1992; Martin, 1996). Indeed an increase in the extent of respiratory CO₂ fixation appears to be a common first step during CAM induction in a number of diverse facultative species (Borland and Griffiths, 1996). Given that the leaf organic Δ of CAM-cycling species are typically in the C₄ range, it would appear that C₄ carboxylation is of minor importance to long-term carbon balance. A model for the partitioning of photosynthetically fixed carbon in source leaves was employed to consider the ecophysiological significance of CAM-cycling in *S. telephium* by assessing the extent to which CAM is induced or enhanced at the expense of growth (Fig. 10; Borland, 1996). The resulting carbon budgets demonstrate the key role for carbohydrate partitioning in the C₃-CAM transition and subsequent magnitude of CAM expression. Starch degradation increases at least twofold with severe drought stress, with the flux of carbon into starch maintained at the expense of the soluble sugar pool. Moreover, under high PFD, the enhancement of CAM-cycling in *S. telephium* conserves water while permitting continued export of carbon and growth, albeit at a reduced rate under conditions of severe drought (Fig. 9). Under low PFD however, the induction of CAM-cycling serves mainly as a maintenance mechanism whereby water is conserved and a positive carbon balance is preserved by recycling carbon skeletons at the expense of export and growth during periods of severe water stress.

### C. CAM and Reproductive Output

For constitutive species, CAM is undoubtedly crucial for growth, survival and reproductive success although relatively few studies have assessed the direct contribution from carbon fixed via PEPC to the construction of reproductive tissues. However, for the prickly pear cactus, *O. ficus-indica*, fruits can exhibit CAM activity, in contrast to the predominantly C₃-like flower buds and young cladodes (Ingleses et al., 1994). For this species, nocturnal CO₂ uptake per unit area of fruit averaged 20% of that of the cladodes for 15 to 45 days after flowering before the activities of PEPC and Rubisco declined in line with fruit chlorophyll content. Thereafter fruit growth was supported by both the bearing and surrounding cladodes.

It might be supposed that in constitutive CAM species and C₃-CAM shifts which are developmentally programmed, such as that exemplified by the well studied *M. crystallinum*, CAM has evolved as a means of partitioning available resources to maximize growth and reproductive output in habitats which are usually seasonally predictable with regard to water supply (Sayed and Hegazy, 1994). Evidence to support this hypothesis was provided by Winter and Zeigler (1992) who showed that, under controlled conditions, salt-stressed plants of *M. crystallinum* which were prevented from fixing CO₂ at night, produced only 10% of the number of seeds produced by plants performing CAM. It was also demonstrated
Fig. 10. Diel carbon budgets for shoots of the C₃-CAM intermediate Sedum telephium grown under a) high PFD-500 μmol m⁻² s⁻¹ or b) low photon flux density—75 μmol m⁻² s⁻¹ and subjected to extended drought stress, as reflected by leaf relative water contents (RWC). Units are mmol C m⁻² d⁻¹. (See Borland, 1996 for full description of model parameters).
that for *M. crystallinum*, C₄ carboxylation makes a direct and substantial contribution to carbon gain in leaves and reproductive structures as determined from carbon isotope discrimination in organic material (Winter and Ziegler, 1992).

A comparison of organic Δ of mature fruits and leaves of the C₃-CAM intermediate *C. minor* and the constitutive CAM *C. rosea* taken from the field indicate distinct partitioning of C₃ and C₄-derived assimilates between vegetative and reproductive growth (A. M. Borland, unpublished). Thus, while the Δ of leaf structural material reflected the relatively greater contribution from carbon fixed by PEPC to seasonal carbon gain in *C. rosea* (12%) compared to *C. minor* (17%), structural material of the fruits from both species showed similar and C₃-like discrimination (18%). Such observations are in line with suggestions that the existence of a transport pool of sugars, synthesized predominantly from Rubisco-mediated carboxylation during Phase IV, will ensure a predominantly C₃ isotopic signature for sugars imported to sinks, regardless of the degree of CAM (Borland et al., 1994). Thus, the unpredictability of resource availability which accompanies the hemi-epiphytic life style of *Clusia* species suggests that while a capacity for the modification of CAM activity undoubtedly maximizes growth and survival, the complete reversibility of such processes and the maintenance of C₃ carboxylation potential throughout these metabolic shifts ultimately ensures reproductive success.

**D. Productivity Indices and a Changing Environment**

Despite the requirement for maintaining two carbohydrate systems and a reciprocating pool of carbohydrates, annual productivities of some CAM species are amongst the highest recorded for any C₃ plants. Pineapple (*Ananas comosus*) is the only CAM plant that is intensively cultivated as a food crop, being grown over some 60 degrees of latitude and a wide range of environmental conditions. Through the use of trickle irrigation which maximizes the contribution from day-time CO₂ uptake during Phase IV, the growth rate and productivity of these plants can approach 35 Mg ha⁻¹ y⁻¹. The optimal spacing and periodic pruning of *O. ficus-indica* to maintain optimal light interception can produce productivities up to 50 Mg ha⁻¹ y⁻¹, exceeding that of the highest producing C₃ crops and trees (Nobel, 1988).

Productivity represents the cumulative, integrative and interactive effects of water supply, PFD and temperature as they relate to the acquisition and allocation of carbon, as detailed in the preceding sections. Nobel and colleagues have modeled the influences of these environmental factors on the productivity of various constitutive agaves and cacti by the calculation of environmental productivity indices. EPI is the product of water, temperature and PFD indices, each with a maximum value of one when that factor does not limit net CO₂ uptake over 24 h (Nobel, 1988). Thus, EPI ranges from zero when one or more factors abolish net CO₂ uptake to one when net CO₂ uptake is maximal. Commonly, EPI is calculated by monitoring 24 h gas exchange. However, the observations by Zotz and Winter (1993a, b; 1994) that the maximum instantaneous rate of net CO₂ uptake can be equated to total 24 h carbon gain could aid the calculation of EPIs for a wider range of species. More recently, a nutrient index has been incorporated into EPI to quantify the effects of soil elements on net CO₂ uptake (Nobel, 1996). This should provide some much needed information on the influence of particular nutrients on the expression of CAM.

By quantifying how changes in various environmental conditions affect productivity, EPI can have a number of practical applications. With a view to optimizing agronomic practice, Nobel (1985) calculated that by irrigating in winter and early spring to induce a water index of one for each month, annual EPI would increase from 0.42 to 0.52 for *Agave fourcroydes* growing in Yucatan, Mexico. In ecological studies, EPI has been used to estimate the growth of particular species along an elevational transect, to investigate plant competition and to estimate plant age in natural populations by calculating the relative growth expected each year (Nobel, 1988).

In the light of climate change, EPI’s have been used to predict both the productivity and geographical regions which CAM plants might successfully exploit (Nobel, 1991). It is generally accepted that over the next century, atmospheric [CO₂] will double alongside changes in temperature and rainfall patterns. In long-term studies with *O. ficus-indica*, doubling [CO₂] resulted in an increased rate of annual dry-matter accumulation of up to 40%, reaching 65 Mg ha⁻¹ y⁻¹, in plots where optimal light interception was maintained by frequent pruning (Nobel and Israel, 1994). Moreover, simulations based on EPI have
indicated that *O. ficus-indica* may presently be effectively cultivated over a substantial fraction of the earth’s surface (Nobel, 1991). It is likely that the continuing development of arid and semi-arid regions of the world will result in an even greater area which CAM crops might successfully exploit.

For CAM plants, in which primary CO₂ fixation via PEPc is commonly thought to be CO₂-saturated at present-day ambient [CO₂], it might be predicted that nocturnal CO₂ fixation will be little affected by elevated [CO₂]. Thus, any influence of elevated [CO₂] on dry-matter accumulation might largely be expected as a result of increased day-time fixation via Rubisco (Winter and Englebrecht, 1994). The substantially increased productivity of *O. ficus indica* in elevated CO₂ described above is therefore particularly unexpected for a plant in which over 80% of net diel CO₂ uptake occurs at night. Moreover, given the high rates of photorespiration measured during Phase IV in some CAM species, which may be related to the low internal conductance to CO₂ (Maxwell et al., 1997, 1998), it will be of considerable interest to determine how constitutive and facultative CAM species respond to elevated CO₂ in their natural habitats.

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Symbols are ignored for indexing, for example, look for $\alpha$-amylase under amylase.

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