

# Electron Transport and Photophosphorylation in Chloroplasts as a Function of the Electron Acceptor\*

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## SUMMARY

Electron acceptors may be divided into three distinct classes on the basis of the nature of the electron transport and phosphorylation processes which accompany their reduction by illuminated chloroplasts.

Class I acceptors are reduced slowly in the absence of phosphorylation. The addition of ADP and  $P_i$  usually increases the rate 2- to 3-fold and up to 1.3 molecules of ATP are formed for each pair of electrons transferred to the acceptor. Uncouplers such as methylamine increase the rate of reduction still further. Most of the electron acceptors heretofore employed in studies of noncyclic photophosphorylation (ferredoxin, ferricyanide, viologens, and flavins) belong to this class. All are ionic or otherwise polar substances with negligible lipid solubilities.

Class II acceptors are reduced rapidly whether or not phosphorylation occurs and the addition of uncouplers often does not increase the rapid rate of reduction. Little or no ATP is formed. Substances such as phenolindophenols belonging to this class have dual functions, serving both as electron acceptors and as uncouplers. They are weak acids, lipid-soluble in their nonionized, protonated forms. Their uncoupling function is enhanced as the pH is lowered and the amount of lipid-soluble acid increases.

Class III acceptors are also reduced rapidly whether or not phosphorylation occurs and uncouplers again cause little or no increase in the rate of reduction. However, when ADP and  $P_i$  are added in the absence of uncouplers a high rate of phosphorylation is observed. The phosphorylation associated with Class III acceptors is usually more rapid than is the phosphorylation associated with Class I acceptors, especially at suboptimal pH. Nevertheless, over a wide range of conditions, the efficiency of phosphorylation in terms of electrons transported ( $P:e_a$ ) is rather precisely half of the efficiency observed when Class I acceptors are reduced. All nonionic, lipid-soluble acceptors we have investigated belong to Class III. Thus the lipid-soluble *p*-benzoquinone is a Class III acceptor but the lipid-insoluble *p*-benzoquinone sulfonate is a Class I acceptor.

From these observations we conclude that electrons are

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transported to Class I acceptors through two sites of phosphorylation whereas the transport of electrons to Class III acceptors utilizes only one of the sites. Presumably lipid-soluble acceptors have access to and accept electrons from carriers which normally transfer electrons between the two sites of phosphorylation.

The Hill reaction, that is, the transport of electrons from water to various electron acceptors by illuminated chloroplasts, has been the subject of innumerable studies during the past 30 years. Nevertheless there seem to have been few attempts to investigate the effects of different acceptors on the nature of the electron transport and the associated phosphorylation processes. It was early recognized that the reduction of indophenol dyes (1-3) was unlike the reduction of other widely used acceptors in several respects. Rates were higher, phosphorylation uncouplers had less effect, and there was much less ATP formation. In the reduction of conventional acceptors such as ferredoxin-NADP or ferricyanide (4), methylviologen, and flavins, no important differences have been observed. All are reduced at low rates in the absence of phosphorylation and at considerably higher rates when ADP and  $P_i$  are added. ATP is formed with comparable efficiency in each instance. Indeed such disparate electron acceptors as ferricyanide ( $E'_0 = +0.36$  volt) and methylviologen ( $E'_0 = -0.44$  volt) are reduced in like fashion; maximum rates of phosphorylating, nonphosphorylating, and uncoupled electron transport are the same with either substance as are the quantum efficiencies of electron transport and of phosphorylation. However, a number of other acceptors including *p*-benzoquinone (5) behave in an anomalous manner and consequently their use has usually been avoided in studies of photophosphorylation. This paper deals primarily with the heretofore neglected topic of the transport of electrons to quinones and quinonediimides.

## METHODS

Leaves of spinach (*Spinacia oleracea* L.) obtained from a local market were ground for 5 to 10 s in a Waring Blendor in a medium containing 0.3 M NaCl, 2 mM  $MgCl_2$ , and 0.04 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-NaOH (pH 7.6). The homogenate was squeezed through several layers of cheesecloth and centrifuged at about  $3000 \times g$  for 4 min. The pellet was resuspended in a medium containing 0.2 M sucrose,

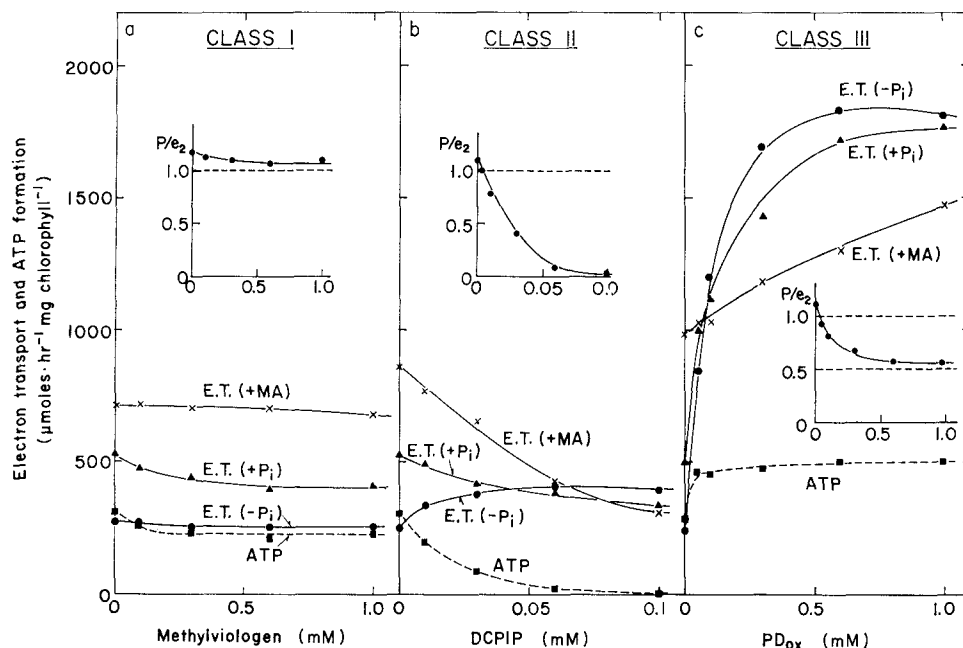


FIG. 1. A comparison of the electron transport and phosphorylation processes with different types of electron acceptor. Reaction conditions: Tricine-NaOH buffer (pH 8.2), 40 mM; potassium ferricyanide, 0.4 mM; ADP, 1 mM;  $K_2HPO_4$  ( $P_i$ ) labeled with  $^{32}P$ , if used, 5 mM;  $MgCl_2$ , 3 mM; methylamine-HCl (MA), if used 5 mM; chlorophyll content of chloroplasts, a, 10; b, 3; c, 20  $\mu g$  per ml reaction mixture; reaction volume, 2.0 ml; temperature, 20°.  $P/e_2$  represents the ratio of the number of molecules

of ATP formed to the number of pairs of electrons transferred to the acceptor.  $PD_{ox}$  represents *p*-phenylenediamine oxidized with 2 eq of potassium ferricyanide immediately before the reaction. The decrease in the amount of excess ferricyanide present in the reaction mixture was used as an indicator of electron transport (E.T.) on the assumption that the other acceptors when reduced would be promptly reoxidized by the ferricyanide.

2 mM  $MgCl_2$ , and 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-NaOH (pH 7.6). The suspension was centrifuged at about  $1200 \times g$  for about 20 s to remove debris, and then centrifuged again at  $3000 \times g$  for 4 min. The washed chloroplasts were finally suspended in a small volume of the same sucrose-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid- $MgCl_2$  medium. All operations were carried out at as near to 0° as possible. The chlorophyll content of the chloroplast suspension was measured by the method of Arnon (6).

In the studies reported here we routinely used the reduction of ferricyanide as an indicator of the reduction of the alternative acceptors being investigated. Thus in most experiments both ferricyanide and the alternative acceptor were present during the reactions. Control experiments indicated that the reoxidation of the reduced form of the alternative acceptor by ferricyanide was sufficiently rapid and complete to make the decrease in the 420 nm ferricyanide absorption a reliable indicator of electron transport. Moreover, in some experiments ferricyanide was omitted from the reaction mixture and the amount of electron transport was measured by titrating the reduced acceptor after the light was turned off. Thus, with oxidized *p*-phenylenediamine as acceptor, rates of electron transport and phosphorylation were unaffected by the presence or absence of ferricyanide. Moreover, the very high rates of reduction of oxidized *p*-phenylenediamine and oxidized diaminodurene represent electron transport and are not artifacts; oxygen is produced at similar high rates and the reaction is totally inhibited by diuron (3-(3,4-dichlorophenyl)-1,1-dimethyl urea).

ATP formation was measured as the incorporation of  $^{32}P$ -labeled orthophosphate after extraction of the unused orthophosphate in the form of phosphomolybdate as described else-

where (7). The rates of phosphorylation and electron transport reported are those observed in the first 15 to 90 s of the reaction.

Oxidized forms of  $PD_{ox}$ <sup>1</sup> and  $DAD_{ox}$  were prepared by adding to the reaction mixture, immediately before the reaction, the amine and 2 eq of ferricyanide (in addition to the excess ferricyanide used to assay electron transport). It is presumed that the oxidation products were primarily the corresponding quinonediimides. However, the oxidized form of *p*-phenylenediamine, in particular, is quite unstable and the oxidation is not completely reversible. Therefore, the chemistry of the preparation that we have designated  $PD_{ox}$  is both complex and uncertain.

Light for illumination of the chloroplast preparations was obtained from the 500-watt incandescent lamp of a slide projector with a 1-liter round bottomed flask filled with dilute  $CuSO_4$  acting as condenser and heat filter. The light was then passed through a broad band red glass filter (transmission >600 nm) before it impinged on the reaction cuvette. Unless otherwise noted the intensity was sufficient to saturate the ferricyanide-reducing system but this intensity may not have completely saturated the more active  $PD_{ox}$  and  $DAD_{ox}$  reducing systems. With the highest concentrations of dichloroindophenol the light intensity was not saturating since the dye absorbed a large part of the incident light. Therefore a small part of the decrease in electron transport observed with increasing amounts of this dye (Fig. 1b) can be attributed to decreasing amounts of actinic light. The low intensities reported in Fig. 4 were obtained by passing the light through a 650 nm interference filter and then

<sup>1</sup>The abbreviations used are:  $PD_{ox}$ , oxidized form of *p*-phenylenediamine, probably mainly *p*-benzoquinonediimide;  $DAD_{ox}$ , oxidized form of diaminodurene, probably mostly duroquinonediimide.

through calibrated fine screens. Incident intensity was measured in absolute units with a wave length independent radiometer (Yellow Springs Instrument Company, Kettering, model 65).

### RESULTS

The characteristics of electron transport and phosphorylation associated with the reduction of conventional electron acceptors are well known. The rate of electron transport is slow when phosphorylation is prevented by the absence of phosphate. Addition of  $P_i$  to the otherwise complete phosphorylation system approximately doubles this slow rate. More than 1 molecule of ATP is formed for every pair of electrons transferred. These phenomena are independent of the concentration of the acceptor, in the range of concentrations shown, and independent of the presence or absence of excess ferricyanide. The slight drop in rates and phosphorylating efficiency seen in Fig. 1a results from the rapid aging of freshly prepared chloroplasts and is not an effect of increasing concentrations of methylviologen. Other substances known to accept electrons in like manner are ferredoxin-NADP, ferricyanide, and flavins. Nearly all studies of noncyclic photophosphorylation have employed one or another of these substances. This category of chloroplast electron acceptors we have designated Class I. All Class I acceptors are highly polar substances with very low lipid solubilities. Therefore it is probable that they cannot approach reaction sites embedded in lipids or behind lipid membranes.

The characteristics of the transport of electrons to 2,6-dichloroindophenol are less well known. The dye increases electron transport rates while almost abolishing ATP formation (Figs. 1b and 3b). This uncoupling effect becomes more pronounced as the pH is lowered. Since lowering the pH converts the blue water-soluble indophenol anion into the red lipid-soluble indophenol acid, it is reasonable to attribute the uncoupling

effect to the indophenol acid. Certainly uncoupling is most pronounced when a significant proportion of the indophenol is present in this membrane-soluble form. The uncoupling effect of the indophenol could be related to its role as an electron acceptor; that is, the indophenol could accept electrons from a carrier which would otherwise contribute electrons to the phosphorylating mechanism. Alternatively, the uncoupling could be a function of the indophenol molecule which is independent of its role as an acceptor. The latter seems probable for two reasons. (a) Many other membrane-soluble weak acids such as fatty acids (8), phenols (9), and the carbonyl cyanide phenylhydrazones (10) also uncouple. (b) Hind and Jagendorf (11) have shown that indophenols inhibited postillumination phosphorylation in chloroplasts; since the indophenol was not added until after the electron transport had ceased there can be no question of its having intercepted electrons. We have assigned such acceptor uncouplers to Class II.

The behavior of quinones and quinonediimides (Class III) as electron acceptors differs strikingly from the behavior of either Class I acceptors or Class II acceptors. This is illustrated in Figs. 1, 2, 3, and 4. With  $PD_{ox}$  or with  $DAD_{ox}$ , electron transport is very rapid and wholly independent of phosphorylation. Nevertheless rapid phosphorylation occurs when ADP and  $P_i$  are present. This phosphorylation is similar in several respects to the phosphorylation associated with the reduction of Class I acceptors; it is prevented by uncouplers, by the energy transfer inhibitor phlorizin, and by the electron transport inhibitor di-

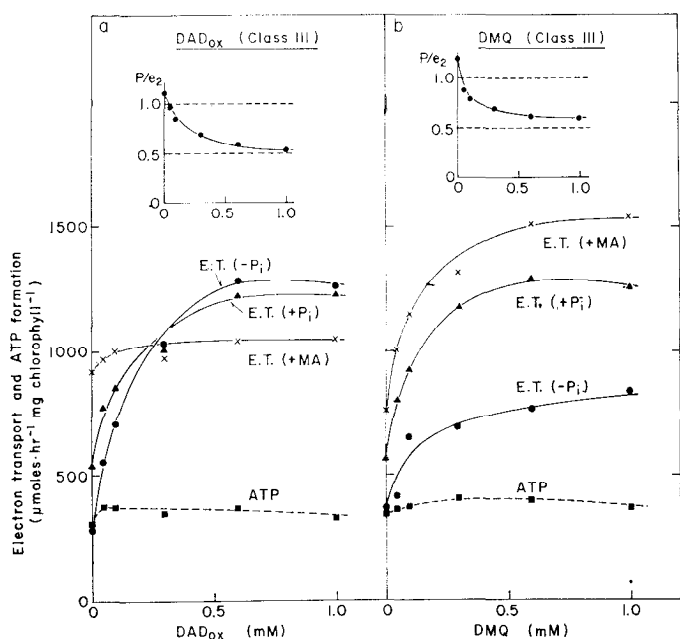


FIG. 2. Electron transport and phosphorylation with  $DAD_{ox}$  and 2,5-dimethyl-p-benzoquinone (DMQ) as electron acceptors. Reaction conditions and abbreviations used as in Fig. 1.  $DAD_{ox}$  was obtained by adding 2 eq of ferricyanide to diamondurene immediately before the reaction.

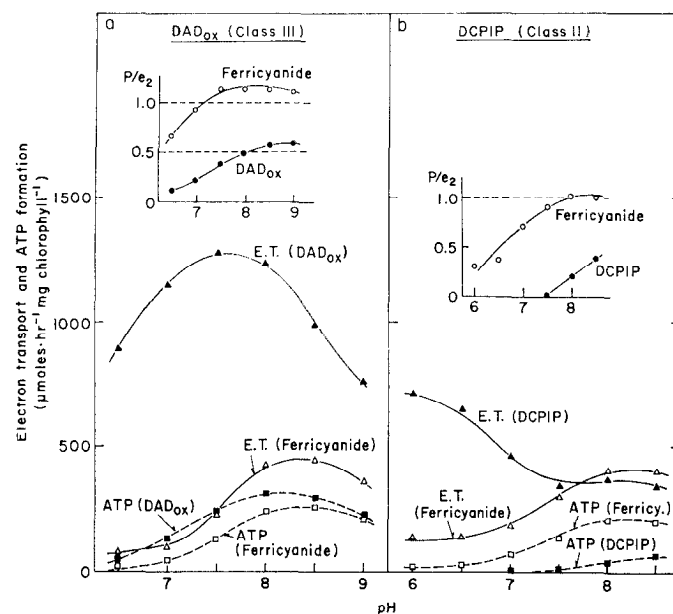


FIG. 3. The effects of pH on electron transport and phosphorylation with ferricyanide,  $DAD_{ox}$  and 2,6-dichloroindophenol (DCPIP) as electron acceptors. Buffer concentration, 40 mM; buffers used: pH 6.0 and 6.5, 2-(*N*-morpholino)ethanesulfonate-NaOH; pH 7.0 and 7.5, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-NaOH; pH 8.0, Tricine-NaOH; pH 8.5 and 9.0, *N*-tris(hydroxymethyl)-3-aminopropanesulfonic acid-NaOH. Chlorophyll content of chloroplasts, a, 20; b, 6  $\mu$ g per ml of reaction mixture. Concentrations of  $DAD_{ox}$ , 0.6 mM, and 2,6-dichloroindophenol, 0.06 mM. Other conditions are as in Figs. 1 and 2. Note that the uncoupling effect of 2,6-dichloroindophenol is correlated with the presence of the red lipid-soluble acid form of the dye which occurs as a major component of the system at pH 6.0.

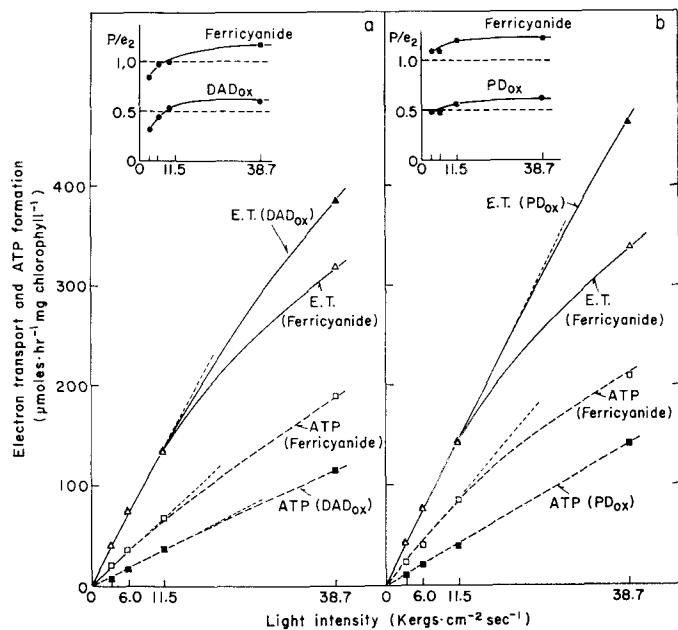


Fig. 4. The relative quantum efficiencies of electron transport and photophosphorylation with ferricyanide, DAD<sub>ox</sub>, and PD<sub>ox</sub> as electron acceptors. Chlorophyll content of chloroplasts, 20 μg per ml of reaction mixture; concentrations of DAD<sub>ox</sub> and PD<sub>ox</sub>, 0.6 mM. Except for light intensity other conditions were as in Figs. 1 and 2. Note that the quantum efficiency of electron transport, as measured by the slopes of the curves at lowest intensities, is the same with all three acceptors whereas the quantum efficiency of phosphorylation observed with ferricyanide as acceptor is twice the quantum efficiency of phosphorylation observed when DAD<sub>ox</sub> and PD<sub>ox</sub> are the acceptors.

TABLE I

*Inhibition of electron transport and photophosphorylation by diuron with Class I and Class III electron acceptors*

Reaction mixture contained Tricine buffer (pH 7.5), 40 mM; chloroplasts with 20 μg per ml of chlorophyll; diaminodurene, 0.6 mM; ferricyanide, 0.4 mM when used alone and 1.6 mM when used with diaminodurene. Conditions otherwise as described in Fig. 1. Rates are in micromoles of ferricyanide reduced or ATP formed per hr per mg of chlorophyll. High levels of inhibitor lower the rate of electron transport to almost the same level with either acceptor but this low rate supports rather less than half as much phosphorylation when the Class III acceptor is reduced.

	Phosphorylating electron transport	ATP formation
Control		
Ferricyanide alone.....	260	123
Ferricyanide + DAD.....	1360	244
10 <sup>-7</sup> M Diuron		
Ferricyanide alone.....	164	72
Ferricyanide + DAD.....	306	50
5 × 10 <sup>-7</sup> M Diuron		
Ferricyanide alone.....	54	11
Ferricyanide + DAD.....	64	4

uron (see Table I). However, phosphorylation with Class III acceptors also differs in several respects from phosphorylation with Class I acceptors. It is much less sensitive to low pH (Fig. 3a) and the rate of phosphorylation is usually markedly higher. But by far the most striking difference is in the efficiency with

TABLE II

*Electron acceptor class as determined by lipid solubility*

Reaction mixture contained Tricine buffer (pH 7.8), 40 mM; chloroplasts with 10 μg of chlorophyll per ml for Experiment I and 20 μg per ml for Experiment II. Other conditions as described in Fig. 1. Values for electron transport and ATP formation are in micromoles per hour per mg of chlorophyll. Ferricyanide and *p*-benzoquinonesulfonate, being anions, are highly polar and therefore insoluble in lipids whereas *p*-benzoquinone is nonionic, relatively nonpolar, and quite soluble in most organic substances. The oxidation-reduction potentials and reactivities of the two quinones are quite similar although the sulfonate is the more oxidizing by about 50 mv. Note that the lipid-insoluble ferricyanide and quinonesulfonate are typical Class I acceptors whereas the behavior of the lipid-soluble *p*-benzoquinone approaches that of a typical Class III acceptor.

Electron acceptor	Assay of electron transport	Electron transport		ATP formation	P:e <sub>2</sub>
		-P <sub>i</sub>	+P <sub>i</sub>		
Experiment I					
Ferricyanide (0.4 mM)	Ferricyanide reduction	240	350	177	1.01
Quinonesulfonate (0.05 mM) + ferricyanide (0.4 mM)	Ferricyanide reduction	216	331	157	0.96
Quinone (0.05 mM) + ferricyanide (0.4 mM)	Ferricyanide reduction	482	614	205	0.67
Experiment II					
Ferricyanide (0.4 mM)	Ferricyanide reduction		289	164	1.14
Quinonesulfonate (0.26 mM) + ferricyanide (0.4 mM)	Ferricyanide reduction		271	128	0.95
Quinonesulfonate (0.26 mM)	Titration of hydroquinone		283	156	1.10
Quinone (0.26 mM) + ferricyanide (0.4 mM)	Ferricyanide reduction		760	285	0.75
Quinone (0.26 mM)	Titration of hydroquinone		637	207	0.65

which the transported electrons are used to generate ATP. The efficiency of phosphorylation (P:e<sub>2</sub>) with Class III acceptors is almost precisely half of the efficiency observed in parallel experiments with Class I acceptors. This is true over a very wide range of conditions with a variety of acceptors used at many different concentrations, at many different pH values, and at many different light intensities. Since this 2:1 difference in efficiency extends to low light intensities, it follows that the quantum efficiency of phosphorylation with Class III acceptors is also half of the quantum efficiency of phosphorylation with Class I acceptors (Fig. 4).

These phenomena associated with the reduction of oxidized *p*-phenylenediamine are not in any way related to the release of uncoupling amounts of ammonia by hydrolysis of the diimide. Such concentrations of ammonia have almost no effect on either electron transport or phosphorylation.

The phenomena associated with the reduction of PD<sub>ox</sub>,



DAD<sub>ox</sub>, and 2,5-dimethyl-*p*-benzoquinone shown in the figures and tables are also encountered with *p*-benzoquinone, 2,6-dichlorobenzoquinone, and the oxidation products of 2,5-diaminotoluene, 4,4'-diaminodiphenylamine, and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. These five substances have not been studied in detail. However, their reduction is also rapid regardless of phosphorylation and phosphorylation rates are high in spite of the apparent uncoupling. It may be, however, that quinones are somewhat intermediate between Class I and Class III. Although quinones also lower the P:e<sub>2</sub> ratio to one-half, they differ from the quinonediimides in that the rate of electron transport in the absence of phosphorylation is considerably lower than is the fully uncoupled amine rate (Fig. 2*b*).

All of the Class I acceptors known are ionic, lipid-insoluble substances and most of the Class III acceptors that we had investigated were very lipid-soluble. Therefore, we suspected that the solubility differences accounted for the differences in the reactions associated with their reduction. To test this hypothesis we compared two closely related substances, *p*-benzoquinone and the *p*-benzoquinonesulfonate ion. Both are oxidants with comparable potentials ( $E'_0$  at pH 8 for *p*-benzoquinone, 0.22 volt; for *p*-benzoquinonesulfonate about 0.27 volt) and both support phosphorylating electron transport in the absence of any other oxidant. However, *p*-benzoquinone is lipid-soluble while the sulfonate is not. As we anticipated, *p*-benzoquinone was found to behave as a Class III acceptor while the sulfonate behaved as a Class I acceptor (Table II).

On the basis of our preliminary survey of chloroplast electron acceptors, this correlation between lipid solubility and Class III behavior seems almost, but not quite, perfect. The oxidized form of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine has finite

but very low lipid solubility whereas it is highly water-soluble. Nevertheless it behaves as a Class III acceptor. On the other hand the reduced form, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, is exceedingly lipid-soluble and only very sparingly water-soluble. It may be that the reduced *N,N,N',N'*-tetramethyl-*p*-phenylenediamine impregnates membranes and thus acts as an electron conduction pathway between the inside and the outside of the thylakoid, or, since the free radical (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine in oxidized form) is unstable, it is possible that the actual acceptor is some other lipid-soluble substance present in catalytic amounts.

#### DISCUSSION

It has long been dogma that the electron transport of the Hill reaction involves a single site of phosphorylation and that the theoretical maximum P:e<sub>2</sub> ratio of noncyclic photophosphorylation is 1.0 (4, 12, 13). However, there are now at least three reasons for believing in the existence of two phosphorylation sites and a theoretically attainable P:e<sub>2</sub> of 2.0.

We have consistently observed P:e<sub>2</sub> ratios above 1.0 during the reduction of ferricyanide by chloroplasts from a variety of plants (14, 15). The same results have been obtained with different methods of measuring phosphorylation and electron transport. The excess over 1.0 is greater than can be accounted for by errors in measurement (7) and probably remains greater than 1.0 even if one allows for some unmeasured cyclic electron transport. Nevertheless it must be kept in mind that, lacking any knowledge of the mechanism of photophosphorylation, we cannot equate P:e<sub>2</sub> ratios to numbers of sites of phosphorylation. A single site involving the movement of a single electron would result in a P:e<sub>2</sub> ratio of 2.0. Indeed the theoretically attainable value of P:e<sub>2</sub> need not even be a whole number if the coupling of electron transport to phosphorylation is somewhat indirect.

Chloroplast preparations, unlike the best mitochondrial preparations, transport electrons at appreciable rates in the absence of phosphorylation. Computation of the theoretical P:e<sub>2</sub> ratio of the phosphorylation process on the basis of the total electron transport implies that no nonphosphorylating electron transport occurs during photophosphorylation—that the addition of ADP and P<sub>i</sub> totally abolishes transport by the mechanism which functions in the absence of ADP and P<sub>i</sub>. Actually the opposite seems to be true. Over a wide range of conditions involving a very wide range of phosphorylation rates ATP formation is proportional to the extra electron transport observed during phosphorylation (15). This can only mean that the nonphosphorylating electron transport continues at an unchanged rate during phosphorylation. When one corrects for the demonstrably constant nonphosphorylating electron transport it becomes apparent that the P:e<sub>2</sub> ratio of the phosphorylation process itself is very close to 2.0.

The phosphorylating efficiency of electron transport with Class I acceptors is rather precisely twice of the efficiency observed when Class III acceptors are reduced. Considering the range of conditions, rates, and substances involved, this is not likely to be fortuitous. It therefore seems probable that two sites of phosphorylation operate when Class I acceptors are reduced whereas only one operates when Class III acceptors are reduced. The correlation between acceptor category and lipid solubility favors this interpretation since it is plausible that lipid-soluble Class III acceptors might have access to and accept

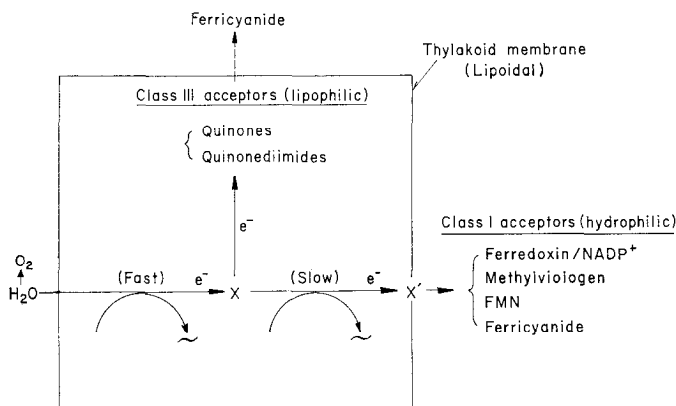


Fig. 5. Model of electron transport and phosphorylation in chloroplasts which could explain the existence of Class I and Class III categories of electron acceptors. It is proposed that quinones and quinonediimides, being lipid-soluble, have access to the intermediate electron carrier  $X$  whereas the conventional lipid-insoluble, ionic electron acceptors do not. It is further proposed that the primary rate-limiting step in the reduction of the ionic Class I acceptors resides in the second coupling site, the transport of electrons from  $X$  to  $X'$ . Therefore the lipid-soluble Class III acceptors are reduced at very high rates but in the process only half as much ATP is formed for a given amount of electron transport. This scheme is clearly oversimplified in that it begs the important question of the nature of nonphosphorylating electron transport which occurs in the absence of ADP and P<sub>i</sub>. It also begs the question of the position of the phosphorylation sites relative to the two photosystems.

electrons from electron carriers which are inaccessible to Class I acceptors (see Fig. 5). Unfortunately this correlation is based on a very limited sample of potential electron acceptors and a much more extensive survey is needed.

The investigations reported here yield little information on the nature or location of the postulated two sites of phosphorylation. Preliminary experiments have not revealed any differences between the two classes of acceptors with regard to the  $K_m$  for  $P_i$ , or sensitivities to inhibitors. If we accept the conventional model of the Hill reaction with its two light reactions in sequence, it is clear on thermodynamic grounds that electron transport from water to Class I acceptors such as methylviologen or ferredoxin must involve both photosystems. Reduction of Class III acceptors would very probably only involve the water-oxidizing Photosystem II (and in fact all such acceptors studied are sufficiently strong oxidants to bypass Photosystem I) but this difference might or might not be reflected in a difference in action spectrum since the photochemistry of the water-oxidizing system could be rate-determining with either type of acceptor. Böhme and Trebst (16) have suggested that one of two photophosphorylation sites may be closely associated with the oxidation of water since the substitution of ascorbate as electron donor inhibited half of the phosphorylation. We are now attempting to investigate these possibilities.

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