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Aquatic CAM photosynthesis: A brief history of its discovery

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ABSTRACT

Aquatic CAM (Crassulacean Acid Metabolism) photosynthesis was discovered while investigating an unrelated biochemical pathway concerned with anaerobic metabolism. George Bowes was a significant contributor to this project early in its infancy. Not only did he provide me with some valuable perspectives on peer review rejections, but by working with his gas exchange system I was able to take our initial observations of diel fluctuations in malic acid to the next level, showing this aquatic plant exhibited dark CO_2 uptake. CAM is universal in all aquatic species of the worldwide Lycophyta genus *Isoetes* and non-existent in terrestrial *Isoetes*. Outside of this genus aquatic CAM has a limited occurrence in three other families, including the Crassulaceae. This discovery led to fascinating adventures in the highlands of the Peruvian Andes in search of *Stylites*, a terrestrial relative of *Isoetes. Stylites* is a plant that is hermetically sealed from the atmosphere and obtains all of its carbon from terrestrial sources and recycles carbon through CAM. Considering the Mesozoic origin of *Isoetes* in shallow pools, coupled with the fact that aquatic *Isoetes* universally possess CAM, suggests the earliest evolution of CAM photosynthesis was most likely not in terrestrial plants.

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1. Introduction

Admittedly it posed quite a challenge to the scientific community to accept the proposition that metabolic patterns observed in an aquatic plant (*Isoetes howellii*, Fig. 1) were indicative of Crassulacean Acid Metabolism (Keeley, 1981). Thinking this was cutting edge science I first submitted the manuscript to the journal *Science* but it received split reviews that resulted in rejection; to paraphrase one reviewer this was 'of profound significance to everyone from molecular biologists to paleoecologists' and the other declared 'this author doesn't understand the first thing about CAM photosynthesis because if he did he would know that CAM is only found in drought adapted plants." I remember sometime later telling George Bowes my frustration with this outcome and he put it in proper perspective by pointing out a similar response when he first tried to publish his discovery of an oxygenase function for what is now known as Rubisco (Bowes and Ogren, 1972). It was somewhat helpful to know that his discovery, one of the more important 20th century discoveries in photosynthesis biology, had a similar experience as my CAM paper, and of course aquatic CAM pales in comparison to Bowes' discovery. Next stop *Nature* but the editor refused to review the ms because it was deemed to be of 'no interest to the readers of *Nature*.' At the time my thinking was that *Nature* would be proven wrong and later I was quite pleased to see that *Nature's* own staff writer Peter Moore concluded this discovery was of significance to *Nature* readers as he wrote a news item in *Nature* highlighting the significance of my CAM discovery that had been subsequently published in the *American Journal of Botany* (see Moore, 1983, 1999).

I recall the first seminar given on this work was in the Biology Department at my alma mater San Diego State University (SDSU) and Professor Chuck Cooper was the first person to shoot up his hand in the question period; to paraphrase, he said 'Jon, you may recall, one thing we instill in our students is that they always ask the right questions. . .*what question!* were you asking when you discovered aquatic CAM photosynthesis?' The answer to that has origins in my graduate studies at the University of Georgia in the southeastern USA, where my ambitious goal was to link genetic polymorphisms in the alcohol dehydrogenase (ADH) enzyme, with functional enzyme kinetics that could explain the physiological function in populations along a gradient from swamps to uplands in the tupelo tree (*Nyssa sylvatica*). This project was soon modified as I learned that the ecological significance of ADH was not well understood. At the time there was vigorous research into alternative metabolic pathways as mechanisms for enhancing flood tolerance in wetland plants. Hochachka and Somero (1973) had

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Fig. 1. *Isoetes howellii* (a seedless vascular plant in the division Lycophyta) in the emergent state after its vernal pool habitat has dried down, exhibiting the classical 'isoetid' growth form of a rosette of cylindrical leaves (photo by J. Keeley).

Fig. 2. Vernal pool on Mira Mesa in San Diego County, California. This mesa had an extensive system of vernal pools but is now covered by track homes.

reported an alternative anaerobic pathway in intertidal bivalves that involved shunting the end-product of glycolysis into organic acids such as malate during anaerobic periods when tides came in, and then recapturing that carbon in the TCA cycle during aerobic periods when the tide went out. R.M.M. Crawford (Crawford and Tyler, 1969) had explored this hypothesis as a flood tolerance mechanism in plants and I tested this idea in swamp populations of tupelo (*Nyssa sylvatica* sensu lato) (Keeley, 1978). However, roots in swamp trees do not experience an alternating aerobic/anaerobic environment as in the intertidal and thus it was little surprise I found no evidence for this pathway in tupelo roots. Upon graduating and accepting a faculty position at Occidental College in Los Angeles I returned to testing the Hochacka and Somera hypothesis. On my trip from Georgia back to California I stopped to visit a former SDSU professor, Robert Hays, whose wife Rachel had received her Ph.D. studying vernal pools at the University of California, Davis, and he pointed out this could be the system where malate metabolism might play an anaerobic role due to the sharp diel patterns of oxygen availability in these shallow pools. Soon upon returning I documented these changes in California vernal pool ecosystems (Fig. 2) with a diurnal shift between aerobic and hypoxic conditions analogous to the intertidal. These rain-fed pools develop over hardpan depressions on mesas and valley bottoms and are shallow and heavily vegetated, and as subsequent studies (Keeley, 1984) showed, they are supersaturated with oxygen (125–150% saturation) during the day but hypoxic (30–40%) at night.

Early on in my career at Occidental College I developed a teaching model of undergraduate labs that explored research issues on as yet unresolved scientific issues, something possible with the outstanding caliber of students. In my physiological plant ecology course we investigated the Hochacka and Somero pathway using vernal pool plants, hypothesizing they were analogous to intertidal organisms exposed to diurnal fluctuations in oxygen availability. Our first field trip was to an area with many vernal pools on mesas in San Diego County of southern California. At the crack of dawn and later in the afternoon we collected the common plant species in the pools and put extracts of the entire plant on ice. Back in the lab extracts were processed for malate levels and we found that one of these aquatic species, *I. howellii*, had a marked diurnal change in malate. Malate levels were very high in the early morning after a night of hypoxic water conditions, followed by the loss of this compound during the highly aerobic daytime conditions, and appeared to "prove" the existence of an organic acid anaerobic pathway. Both the students and I were very excited by this apparent 'discovery.'

Subsequently I submitted a grant to the American Philosophical Society to follow up on these observations and with a small amount of funding I set out the next spring to test my first hypothesis, which was that this anaerobic pathway would be largely concentrated in the densely packed tissues of the corm and less so in the leaves with large lacunae or air chambers. On my return to the lab and processing the samples I learned quickly that there was a major problem with this hypothesis since the diurnal changes in malate levels only occurred in the leaves! An additional clue that suggested CAM photosynthesis was that we routinely assayed for protein levels since the Lowry test is a simple assay for students to learn. What we discovered was that, after extraction and centrifugation, the leaf extract had no protein in the morning and abundant protein in the afternoon. This was rather perplexing until reading in the early CAM studies on photosynthesis that similar patterns had been observed in pineapple leaves; until researchers recognized

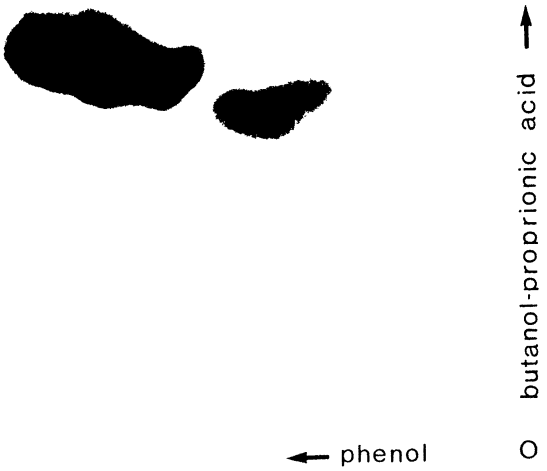

Fig. 3. Autoradiograph of labeled products after 1 h of dark $^{14}CO_2$-fixation. The largest spot is malic acid, the smaller is citric acid. The origin is the lower right hand corner, the horizontal solvent was phenol and the vertical solvent was butanol–propionic acid (from Keeley, 1981).

that high acidity of early morning extracts caused the precipitation of proteins (Sideris et al., 1948). Soon, based on changes in titratable acidity, it was apparent that in *Isoetes* it was malic acid accumulating overnight (and as long as extracted in highly buffered solutions there was no change in protein concentration).

2. Adaptive significance of CAM

One of the important tests of CAM photosynthesis is not just that there is an overnight accumulation of malic acid but that it is the immediate product of carbon fixation in the dark and that this carbon is retained in malic acid during the dark period. Once these experiments with ^{14}C were completed (Fig. 3) I felt confident enough to publish the first paper on aquatic CAM photosynthesis (Keeley, 1981).

Of course this interesting observation required much more elaboration on its biochemical pathway, physiological functioning, ecological role and global distribution. I systematically set out to investigate each of these aspects. Not being equipped for aquatic plant gas exchange work at the time I got in touch with George Bowes at the University of Florida as he and his student Michael Salvucci had data suggesting different photosynthetic mechanisms in submerged aquatic plants (Salvucci and Bowes, 1983), and whose lab was equipped to investigate aspects of photorespiration and other characteristics of aquatic plant gas exchange. I packed up a supply of living *I. howellii* and spent two weeks in George's lab at the University of Florida. In the Hydrilla plants they had been studying there was a weak accumulation of acids overnight but no net CO_2 uptake. I remember my first night of experiments in the lab when George headed home for dinner he remarked something to the effect 'don't be discouraged if you don't find any dark CO_2 uptake.' However, my work with George Bowes demonstrated that, in association with overnight increases in malic acid, there was net CO_2 uptake in the dark, the *sine qua non* of CAM. However, this work also showed that given sufficient CO_2 in the water *Isoetes* was capable of net CO_2 uptake in the light as well (Keeley and Bowes, 1982). Since some terrestrial CAM plants likewise exhibit low level CO_2 uptake in the early morning this was not entirely inconsistent with the patterns in terrestrial CAM plants.

I applied for and received my first NSF grant followed by several more over the next 10 years focused on a fuller investigation of this surprising discovery. With several outstanding undergraduate students including Darren Sandquist, Teresa Swida, Geoff Busch, Bryce

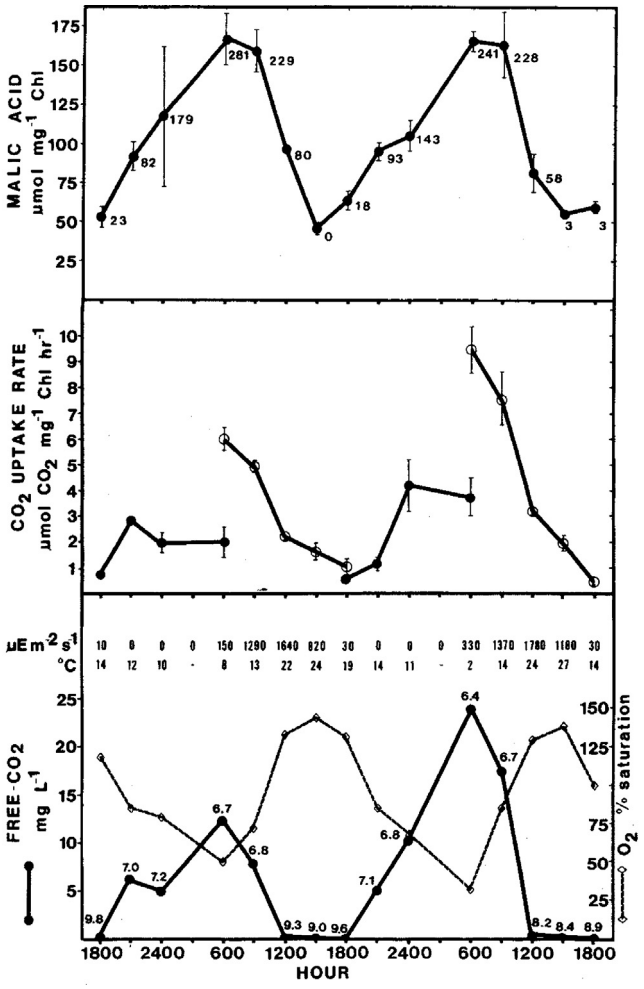

Fig. 4. (Top) malic acid levels in leaves of *Isoetes howellii* (numbers above line are titratable acidity, µEq mg^{-1} chlorophyll), bars indicate ±1 SE, $n = 3$); (middle) carbon assimilation measured with ^{14}C in the dark (closed circles) and light (open circles); (bottom) oxygen and carbon dioxide in the pools; since the pools are rain-fed and largely unbuffered, diurnal changes in CO_2, which converts to carbonic acid in unbuffered solutions, resulted in pronounced changes in water pH, indicated with numbers above the curve. Factors driving this marked diurnal change in pool chemistry were the shallow nature of the pools, and thus high mass of photosynthetic material relative to water volume, and the high diurnal change in pool temperatures of 15–20 °C at night and 30–35 °C during the day, which impacted CO_2 solubility. The dense vegetation with high photosynthetic demand during the day and with high respiratory activity overnight are thought to be the primary drivers of marked changes in CO_2 and O_2 in the pools.

Morton, Cindy Miller Walker and many others, I set out to examine more closely the ecological aspects of aquatic CAM by monitoring cycles of water chemistry and other physical parameters and associate them with patterns of metabolic changes in *Isoetes*.

These studies demonstrated marked diurnal changes in water chemistry in the field, in particular a diurnal cycle of CO_2 availability in the water, which was high at night and essentially absent during the latter half of the day (Keeley, 1983a,b; Keeley and Busch, 1984). In parallel, malic acid levels rose in the leaves at night as ambient CO_2 increased and declined during the day in parallel with the decline in ambient CO_2 (Fig. 4). Using ^{14}C we showed that in the field there was significant CO_2 uptake overnight and although high in early morning it declined to almost nil carbon uptake in late afternoon, in parallel with the decline in ambient CO_2 (Fig. 4). Laboratory studies demonstrated an inability of *I. howellii* to take up bicarbonate (Keeley, 1983a,b) and therefore a complete reliance on CO_2. Thus for *I. howellii* in the field an external carbon source for photosynthesis was absent during much of the day (Fig. 4). This

diurnal cycle in ambient CO_2 provided a selective basis for the evolution of CAM photosynthesis in this aquatic plant. In brief, elevated ambient CO_2 at night is taken up through the CAM pathway and stored as malic acid (Fig. 4). During the day as CO_2 in the pools declines, uptake of CO_2 declines, leading to an internal 'starvation' of CO_2, which induces the decarboxylation of malic acid to generate an internal source of CO_2 for photosynthesis. This is not unlike the situation with terrestrial CAM plants except that the diurnal cycle in CO_2 availability is driven by changes in stomatal opening and closing and not by changes in ambient CO_2 availability.

Nighttime acidification and daytime de-acidification tracked ambient CO_2 levels and this was relatively fine-tuned such that on over-cast days ambient CO_2 levels were not always depleted during the day and this was correlated with less de-acidification (Keeley and Busch, 1984). ^{14}C tracer studies showed that overnight carbon uptake was stoichiometrically consistent with levels of malic acid accumulation and in the light, carbon from malic acid rapidly accumulated in early products of C_3 photosynthesis (Keeley, 1996).

Anatomical studies showed that these patterns were not correlated with stomatal opening and closing; stomata were present on underwater leaves but were non-functional and CO_2 uptake day and night was due to passive diffusion driven by ambient levels of CO_2 in the water. Thus, carbon uptake occurred both at night and during the morning before ambient CO_2 levels were completely consumed by the associated pool flora. Quantitative studies showed that dependent on conditions, dark fixation accounted for a third to half of the total 24 h carbon uptake (Keeley, 1998).

When water levels dropped as the vernal pools dried down and leaves were exposed to the atmosphere, overnight carbon uptake and malic acid accumulation were lost on a cell by cell basis and photosynthesis was dominated by direct daytime CO_2 incorporation into the C_3 pathway. Due to the high water vapor deficit of air, leaves rapidly developed a thick impervious cuticle and CO_2 entry in the leaves was now controlled by functional stomata (Keeley and Busch, 1984).

One characteristic not consistent with terrestrial CAM species is the ratio of stable isotopes of carbon, $^{13}C/^{12}C$, or $\delta^{13}C$, which arises from discrimination between $^{13}CO_2$ and $^{12}CO_2$ (e.g., Griffiths, 1992). In terrestrial plants it is a reliable marker for CAM, C_4, and C_3 photosynthesis. However, in aquatic plants this ratio is largely indistinguishable among the three photosynthetic pathways. The discrimination between ^{13}C and ^{12}C isotopes in terrestrial CAM and C_4 species is the result of internal diffusional limitations that are not experienced by C_3 species (Griffiths, 1992). In aquatic systems the largest diffusional limitations are due to the water environment and affect all species regardless of photosynthetic pathway (Keeley and Sandquist, 1992).

3. Distribution of aquatic CAM

With growing evidence that *I. howellii* was an aquatic CAM plant, attention turned to how widely distributed this pathway is, and various labs around the world independently joined in this effort. *Isoetes* has a global distribution and CAM was found in vernal pool species in southeastern USA, Australia, South Africa, Chile, and the Mediterranean basin (Keeley, 1998). This taxon is also widely distributed in lakes, but primarily under oligotrophic conditions such as lakes in the Sierra Nevada alpine, upper Mid-west, Scandinavia, Scotland and tropical alpine environments (Keeley et al., 1981, 1983; Boston and Adams, 1983; Madsen, 1985; Keeley, 1998). In these species CAM is not driven by diurnal cycles in CO_2 availability as in vernal pools, but rather by the extraordinarily low levels of CO_2 in the water. Under these conditions CAM doubles the amount of time for capturing carbon through both dark CO_2 uptake and light CO_2 uptake (Sandquist and Keeley, 1990).

In the genus *Isoetes*, which numbers in the hundreds, there is a small handful of terrestrial species that are never submerged in water. Two such species *I. nuttallii*, largely Californian, and *I. butleri*, from the southeastern USA, occur in substrates that are vernally moist but are never submerged. Neither are CAM, and CAM cannot be induced by extended submergence (Keeley, 1983a,b). In short, CAM is clearly a pathway associated with the aquatic environment in *Isoetes*.

Our lab did an intensive study of the California vernal pool flora and found that of the several dozen species only one other taxon exhibited evidence of CAM, *Crassula* (*Tillea*) *aquatica* (a diminutive annual in the Crassulaceae, the nominate CAM family). Studies showed that as with *I. howellii, C. aquatica* also lost CAM upon emergence from the pools. Despite its phylogenetic affinity to drought adapted CAM species, when exposed to the drying conditions out of water, CAM was of selective value under water but not in response to emergent conditions. Consistent with this pattern, *C. erecta* is a terrestrial annual that often occurs on exposed mounds on the periphery of vernal pools and it exhibits all of the same characteristics of the terrestrial *I. nuttallii*, i.e., not CAM, and CAM cannot be induced by submergence. This pattern extends to other annual *Crassula* species found throughout the world (Keeley, 1998). Terrestrial ephemeral *Crassula* are C_3 for their short life span and CAM appears to provide little adaptive value. But for those ephemeral *Crassula* that occur as submerged aquatics, CAM has adaptive value.

Aquatic CAM is not widely distributed and only a few other species have this pathway; e.g., *Littorella uniflora* (Plantaginaceae) from high latitude oligotrophic lakes (Keeley and Morton, 1982) and lowland lakes of Scotland and Scandinavia (Aulio, 1985; Madsen, 1985). As with other aquatic CAM taxa, terrestrial

Fig. 5. Drs. Barry Osmond (left) and John Raven admiring our collection of *Stylites andicola* in a hotel room in Junin, Peru, circa 1982 (photo by Jon Keeley).

Fig. 6. *Stylites andicola* embedded in peat showing nearly 95% of the biomass occurs underground (coin is ~3 cm in diameter) collected from the perimeter of Lago Junin, Peru.

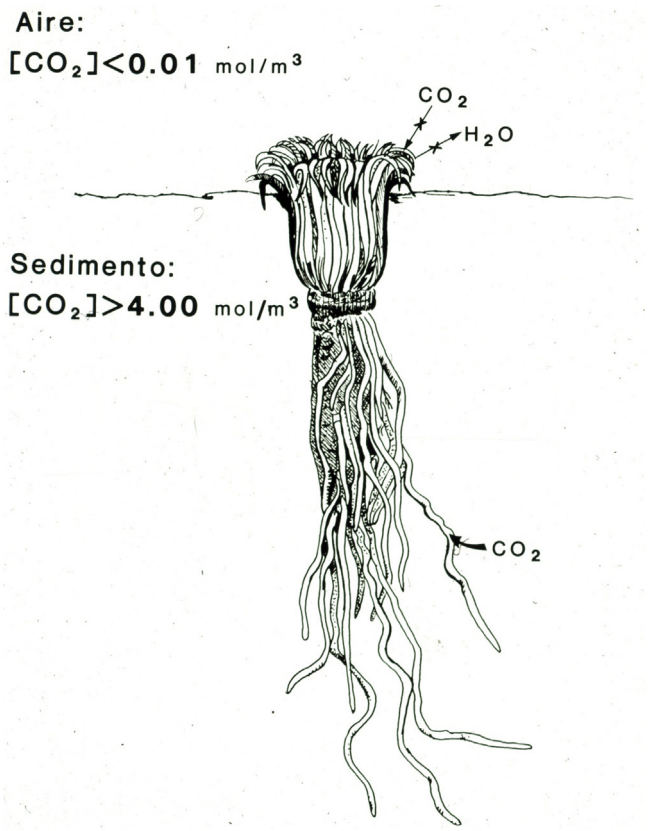

Fig. 7. Schematic view of carbon sources and uptake by *Stylites andicola* (from a talk given in Spanish at the IV Congreso Latinoamerico de Botanica, Medillin, Colombia, I986).

populations of *L. uniflora* are not CAM (Aulio, 1985). *Littorella* has the similar isoetid morphology as found in *Isoetes* (Fig. 1), as does *Sagittaria subulata* (Alismataceae), which also appears to be an aquatic CAM plant. A few other aquatic taxa show low level changes in acidity that may be indicative of CAM (Keeley, 1998).

3.1. *Stylites*

At the 1980 Botanical Congress in Sydney I had lunch with Barry Osmond, whom I had only previously corresponded with but never met. I was somewhat surprised by his first question at lunch, to paraphrase 'What do you think about CAM in the earliest land plants.' He elaborated that he had just that morning been in a workshop moderated by John Raven on the physiology of the earliest land plants and one of the observations reported was that epidermal impressions of the earliest land plants apparently lacked stomata. This prompted the thought that perhaps these plants were absorbing carbon from the sediment and recycling it internally with the CAM pathway. As I remember my response was something along the line of 'Errrr. . .. I don't know what to think of that idea . . . but, I know of a species related to *Isoetes* that could be used to test it.' I was thinking specifically of *Stylites*, a sister genus of *Isoetes* (and by some taxonomies synonymous with *Isoetes*). I knew from the literature it was a terrestrial species that lacked stomatal pores. This taxon is a localized endemic to very high elevations in the Peruvian Andes. Barry's immediate response was (to paraphrase) "Well, let's go get it and find out!" Thus, at the Sydney Congress a plan was hatched for Barry, John, and I to meet in Peru. Being a new faculty member I had limited funding for such spontaneous searches but had made contacts with physiological ecologist Jim Teeri (University of Chicago), someone with a knack for finding philanthropic donors. Over dinner one evening at a conference I told him about these ideas and excited him to the extent that he offered to pay for my trip and I gratefully accepted. John Raven and I met in the airport in Bogota Colombia for the final leg of our journey to Lima, Peru where we met Barry Osmond. We rented a car and after an interesting evening on the town, we headed the following morning into the high Andes to the type locality for *Stylites andicola*; raised bogs at the periphery of Lago de Junin at 4100 m. After a couple days of searching we found *Stylites* (Fig. 5). Particularly striking to us was that leaves only protruded from the sediment a short distance and nearly 95% of the biomass was underground (Fig. 6). Field studies showed diurnal changes in CAM-like acidity patterns and when samples were later assayed in the lab this was shown to be due to malic acid.

Stylites grows on high mounds of peat material and we collected many specimens and returned them to my lab at Occidental College. ^{14}C studies in split chambers showed that the leaves of this species failed to take up CO_2 and these highly cutinized non-stomatous leaves were in essence hermetically sealed from the atmosphere (Fig. 7). However, the roots took up carbon and this labeled carbon rapidly accumulated in the leaves. Calculations done by Park Nobel at UCLA supported the conclusion that this transport was through mass action in airspaces in the roots and stems. This paper was accepted by *Nature* (Keeley et al., 1984).

An indirect test of this hypothesis was conducted by Sternberg et al. (1985) by measuring the natural abundances of ^{14}C. This study took advantage of the substantial changes in atmospheric ^{14}C resulting from atmospheric nuclear bomb testing during the middle of the 20th century; the ^{14}C content of the atmosphere nearly doubled in the early 1960s, but has declined since the ban

Fig. 8. The author with two substantial aquatic *Isoetes* from Lago Khara Koto, Boliva showing the substantial portion of the leaves are achlorophyllous due to being buried in the carbon-rich sediment.

on atmospheric testing. Measurements taken in the early 1980s of ^{14}C showed levels in the leaves of *Stylites* were much higher than contemporary atmospheric levels and bore a strong resemblance to levels in the peat moss, which had been generated by photosynthesis many decades earlier when atmospheric ^{14}C was high due to nuclear testing. To this day Barry Osmond in his memoirs describes *Stylites* as "the most bizarre photosynthetic system in my experience" (Osmond, 2014).

It is important to note that carbon uptake from roots had previously been well documented for some aquatic plants, in particular for *Isoetes* and others with the isoetid growth form (Wium-Anderson, 1971; Sondergaard and Sand-Jensen, 1979). This also appears to be the case for some of the *Isoetes* in Andean lakes (e.g., Fig. 8) as the extensive achlorophyllous leaf bases are thought to enhance CO_2 uptake from the sediment (Pedersen et al., 2011). The presence of this trait in a terrestrial species such as *Stylites* raises interesting questions about the origin of this trait.

4. Early evolution of CAM

Isoetes is sometimes described as a fern-ally to emphasize its relationship with the non-flowering Lycophyta. It has affinities with taxa present in the early Mesozoic and the genus dates to the Triassic (Keeley, 1998). In contrast to these aquatic CAM plants, most all contemporary terrestrial CAM plants are in the Anthophyta and are in lineages that mostly date to the early Tertiary. Selection pressure for the evolution of CAM is thought to have been driven by changes in atmospheric carbon and geological periods differ in their favorability for CAM. Based on geological changes in atmospheric CO_2, CAM is widely considered to be a relatively recent innovation in plant evolution (Raven and Spicer, 1995). However, there are compelling reasons to believe that the earliest CAM plants were aquatic *Isoetes*. Although atmospheric conditions during the early Triassic were unfavorable for terrestrial CAM plants (Raven and Spicer, 1995), carbon limitations in the aquatic milieu are not closely tied to atmospheric conditions (e.g., Fig. 4). *Isoetes* evolved in the early Triassic in small seasonal pools and there is no reason to believe that the diurnal fluctuations in carbon availability would have been qualitatively different from that seen in contemporary pools. This and other observations support the notion that CAM may have first appeared in these aquatic plants a hundred million years before in terrestrial species (Keeley, 1998).

5. Conclusions

Aquatic CAM plants provide a challenge to understanding how similar biochemical pathways could evolve in disparate environments. A useful construct is keeping separate proximal and ultimate answers to this question. Proximal answers focus on 'how' organisms respond functionally and ultimate answers focus on 'why.' Both terrestrial and aquatic CAM plants are responding to limited internal daytime CO_2 availability, in an environment where nighttime CO_2 levels are higher. In terrestrial CAM plants low CO_2 during the day arises from stomatal closure, due to the high water deficit of arid and semi-arid environments, whereas in aquatic CAM plants it is the result of depletion of ambient CO_2. Thus, proximally, both aquatic and terrestrial CAM plants have selected the same biochemical pathway to provide the same benefit; supplying CO_2 at the active site of photosynthesis during the day when external sources are unavailable, due either to depletion in the ambient environment or stomatal closure. However, ultimately selection for this pathway has derived from profoundly different environmental cues in aquatic and terrestrial environments. In the latter it arose in response to arid conditions that selected for daytime stomatal closure and in the former to low CO_2 availability in the ambient aquatic environment.

Acknowledgments

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