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ISOETES HOWELLII: A SUBMERGED AQUATIC CAM PLANT?[1]

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ABSTRACT

In the leaves (but not corms) of the submerged aquatic plant *Isoetes howellii,* malic acid concentration fluctuates from 1–3 mg g^{-1}FW in the evening to 7–13 mg g^{-1}FW in the morning. Associated with this is a change in pH (a.m. pH 3–4 vs. p.m. pH 5–6) and titratable acidity (75–200 μeq g^{-1}FW change in acidity between morning and evening) of the plant extract. $^{14}CO_2$-fixation experiments indicate that carbon is fixed in both the light and the dark, though the amount of carbon fixed in the light is more than double that fixed in the dark. Autoradiographs show 89% of dark-fixed CO_2 ends up in malic acid and the remainder in citric acid, whereas these two acids constitute less than 5% of the light-fixation products. It is suggested that CAM metabolism in this aquatic species may be related to the lower availability of CO_2 for photosynthesis during the day than during the night in its aquatic environment.

DARK-FIXATION of CO_2 is known to occur in roots, stems, and leaves of many plants both terrestrial and aquatic (e.g. Jacobson, 1965; Webb and Burley, 1965; Ting and Dugger, 1966; Willenbrink et al., 1979). In photosynthetic tissues of drought-adapted succulents known as CAM (crassulacean acid metabolism) plants, dark-fixation of CO_2 substantially contributes to the total carbon gain of the plant (Kluge and Ting, 1978). In CAM plants, CO_2 is fixed at night, through β-carboxylation of phosphoenolpyruvate, into organic acids (largely malic acid), which are stored in the cell vacuole until morning. During the day, these organic acids are removed from the vacuole, decarboxylated, and the CO_2 released is incorporated into carbohydrates via the Calvin (C_3) pathway of photosynthesis.

Crassulacean acid metabolism is unique in that it combines *all* of the following characteristics: 1) dark-fixation of CO_2 takes place in photosynthetic tissues; 2) C_4 acids are the first stable products of dark CO_2-fixation; 3) malic acid is quantitatively the most important of these organic acids; 4) malic acid, rather than malate, accumulates overnight and thus is sequestered in the vacuole, and 5) the diurnal cycle of nighttime acidification and daytime deacidification involves a diurnal fluctuation of 75–200 μeq g^{-1} fresh weight.

The physiological significance of CAM is that it generates an internal CO_2 source that can be utilized during the day. In drought-adapted succulents, CAM serves an important ecological function in that it allows Calvin-type photosynthesis to proceed with stomates closed, thereby conserving water. Stomates open at night when the vapor pressure deficit is much lower than during the day. Thus, a diurnal pattern of open and closed stomates is generally associated with CAM, though under certain conditions many CAM plants (in addition to or in place of dark CO_2-fixation) also fix externally derived CO_2 directly into carbohydrates during the day (Kluge and Ting, 1978). Osmond (1978) contends that CAM is more appropriately described in physiological rather than ecological terms and in this context it is conceivable that the same metabolic pathway may play more than one ecological role. Recent work in my laboratory indicates CAM apparently occurs in certain submersed aquatic plants, where it undoubtedly plays a role other than conserving water.

Isoetes howellii Engelmann is an aquatic or amphibious "lower vascular plant" in a family (Isoetaceae) distributed world-wide in aquatic (freshwater) or waterlogged habitats (Pfeiffer, 1922). *Isoetes* species are morphologically quite similar; all possess quill-like leaves arising from an underground corm, and each leaf has four longitudinal air canals, separated from one another by septa and surrounded by a peripheral wall of green tissue. Sporangia are produced at the base of these leaves, which technically are considered microphylls (single unbranched veins and no leaf trace gap in the stem stele). Based on these characteristics, members of the Isoetaceae usually are classified in the subdivision Lycopsida, an ancient

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group extending back to the Devonian (Foster and Gifford, 1974).

Isoetes howellii is distributed throughout California and other western states in temporary pools (Munz, 1959) and is often a component of the unique California Vernal Pool Ephemeral Community (Thorne, 1976). This community is found in the winter and spring in vernally wet pools which form in shallow basins underlaid by an impervious hardpan. Because of the Mediterranean climate, the pools are filled by winter rains and dry out during the summer drought. Field and laboratory experiments with *I. howellii* were conducted during the spring of 1979 on plants from vernal pools on Miramar Naval Air Station, San Diego, California.

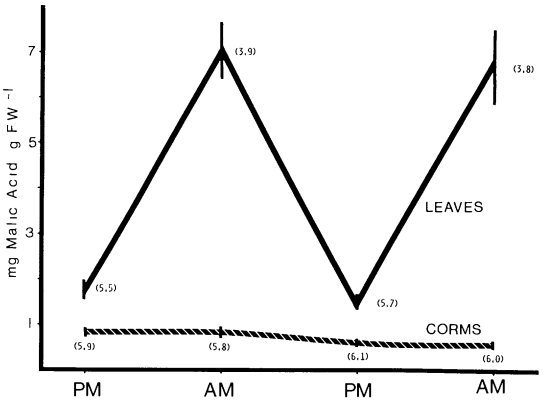

Fig. 1. Malic acid concentration (mg per g fresh weight) in leaves and corms of *Isoetes howellii* from Miramar Vernal Pools, at 6:00 a.m. and 6:00 p.m. (±30 min) April 6–8, 1979. Extract pH is in parentheses. Each point is the mean of six samples; vertical lines are ±1 SE.

METHODS—All experiments were done on plants growing submerged. In the field, six plants were collected within 30 min of 6:00 a.m. and 6:00 p.m., washed in distilled water, blotted dry, weighed, ground in a blender (run with a portable generator) with distilled water, filtered through cheesecloth, and centrifuged at low speed. The pH of this supernatant was measured with a portable pH meter, then a sample of supernatant was deproteinized, placed on ice, returned to the lab, and assayed enzymatically for malic acid (Bergmeyer, 1974, p. 1585–1589). The remaining extract was returned to the lab, centrifuged, and a sample of supernatant assayed for protein with a modified Lowry test (Bergmeyer, 1974, p. 172–174) and with the Bio-Rad Laboratories assay (Tech. Bull. No. 1051), the results of which were very comparable. In later experiments, titratable acidity was determined as described by Siders et al. (1948).

$^{14}CO_2$ studies—Relative $^{14}CO_2$-fixation in the light and dark was measured twice at night (10–11:00 p.m.) and twice in the morning (10–11:00 a.m.). For each experiment, leaf material, either intact or cut into 2-cm sections, was placed in 50-ml round bottom flasks with 10 ml of distilled water. Four dark-fixation flasks were covered with foil and incubated along with four light-fixation flasks in a shaker waterbath at 25 C for 15 min, after which 200 μCi of 2 mM $NaH^{14}CO_3$ was added. All flasks were tightly stoppered and incubated for 1 hr. Light-fixation flasks were exposed from the side to incandescent light at an intensity of 2000 $\mu E\ m^{-2}\ sec^{-1}$ (PAR). Experiments were ended by adding boiling 80% ethanol. These were acidified to remove unfixed ^{14}C and extracted by boiling for 45 min. Extracts were counted on a scintillation counter (Beckman LS100C) and, after correction for efficiency, expressed as disintegrations per min. Various buffers and light intensities were utilized, but the conditions described above gave maximum light- and dark-fixation. To check for interference by externally attached microbes, leaves rinsed in 10% Clorox were compared with leaves rinsed in distilled water; no difference was observed.

The ^{14}C-labelled products were separated with 2-way paper chromatography from which autoradiographs were developed as described by Pedersen, Kirk and Bassham (1966). Separation in the first dimension was with phenol for 30 h and the second dimension was with butanol-proprionic acid for 24 h.

RESULTS—Figure 1 shows diurnal changes in malic acid concentration between April 6 and 8 for plants in the field. Leaves (but not corms) showed a 4–5-fold increase in malic acid overnight with morning concentrations typical of many succulent CAM plants. This experiment has been replicated numerous times and the only difference observed is that the magnitude of diurnal fluctuation in malic acid concentration (in submerged plants) tends to increase through the season, reaching levels of over 10 mg g^{-1}FW in some plants in June (p.m. 3 to a.m. 13: mg g^{-1}FW). These subsequent experiments also showed a diurnal fluctuation in titratable acidity of the same magnitude as seen for malic acid. Diurnal changes in titratable acidity also varied through the season from ca. 75 μeq g^{-1}FW early in the season to over 200 μeq g^{-1}FW late in the season.

Presumably, the malic acid produced overnight is sequestered in the cell vacuole. This is suggested by the observation that the acidity of the plant extract in the morning (Fig. 1) was

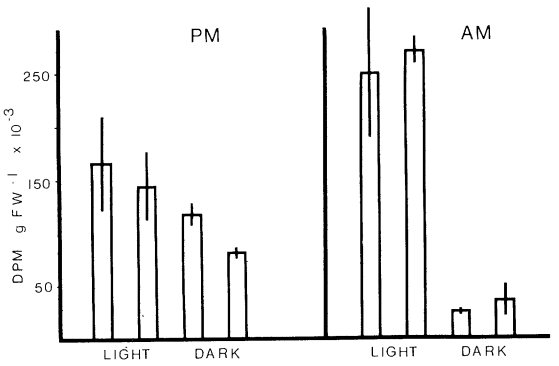

Fig. 2. Relative $^{14}CO_2$-fixation in the light and dark. Both light- and dark-fixation were measured twice at night (PM) and twice in the morning (AM). Bars represent the mean of four replicates, vertical lines indicate ±1 SE.

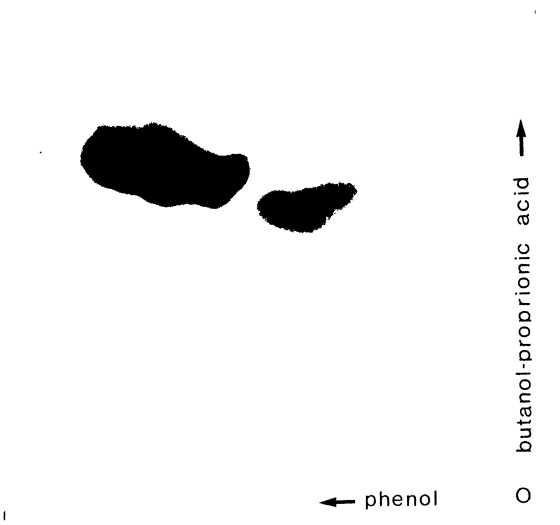

Fig. 3. Autoradiograph of labeled products after 1 hr of dark $^{14}CO_2$-fixation. The largest spot is malic acid, the smaller is citric acid. The origin is the lower right hand corner, the horizontal solvent was phenol and the vertical solvent was butanol-propionic acid.

sufficient to precipitate most of the protein. Extracts prepared in the morning had only 2% of the protein which was in solution in the evening extract. The low protein concentration in morning leaf samples was apparently due to precipitation by malic acid upon disruption of the cells, as evidenced by the high protein concentration obtainable by resuspending the morning leaf pellet. In conjunction with this, photosynthetic cells of *I. howelli* have large vacuoles (Paolillo, 1963).

In order to ascertain whether or not nighttime malic acid production results from dark fixation of CO_2, $^{14}CO_2$-fixation experiments were conducted. *Isoetes* were transplanted to artificial pools on campus and the water level maintained just above the tips of the leaves. *Isoetes* in these pools showed a diurnal acidification-deacidification cycle identical to plants in the field. A comparison of $^{14}CO_2$ uptake by *Isoetes* leaves in the light and the dark is shown in Fig. 2. It is apparent that *Isoetes howellii* is capable of fixing CO_2 under both conditions. Leaves are capable of assimilating substantial amounts of $^{14}CO_2$ in the dark, relative to the light. Carbon dioxide uptake in the light was greatest in the morning, at which time dark fixation was greatly inhibited. This morning inhibition of dark fixation has been observed in CAM plants (Kluge and Ting, 1978) and is thought by some to arise from inhibition of carboxylation by elevated levels of cytoplasmic malic acid.

To determine to what extent dark-fixed carbon is retained, plants were labeled in the dark for 1 hr after which one set was killed and several others flushed of remaining unfixed $^{14}CO_2$, and then incubated in the dark. After 1 hr in a $^{12}CO_2$ atmosphere, 97% of the dark-fixed label was still fixed and 89% was still fixed after 3 hr.

In order to show that the CO_2 fixed in the dark is related to the diurnal acid metabolism, the ^{14}C-labeled products were separated with 2-way paper chromatography from which autoradiographs were developed. Figure 3 shows the autoradiograph of 1-hr, dark-fixation products. The darkest spot was eluted and shown enzymatically to be malic acid. The other product was identified as citric acid by cochromatographing malic and citric acids and determining their relative positions. Both spots were eluted and counted, showing that 89% of the label was in malic acid. Subsequent dark CO_2-fixation experiments indicate occasionally a trace of label in aspartate.

The autoradiographs of 1 hr light-fixation indicated a distribution of radioactivity in many products not unlike that found in C_3 photosynthesis; most of the label was recovered in carbohydrates and amino acids with only 5% in malic and citric acids. Short-term labeling experiments showed that after 30 sec of light-fixation, predominantly 3-PGA and phosphorylated sugars were labeled, further indicating operation of the C_3 cycle in the light.

To evaluate the fate of dark-fixed carbon in the light, a 1 hr pulse of $NaH^{14}CO_3^-$ in the dark was chased up to 1 hr in the light in a ^{14}C-free environment. Under short term conditions, up to 10 min, all the label remained in malic and citric acids. After 1 hr, 98% of the label was still retained by the plant but ca. 35% was in 3PGA and carbohydrates.

DISCUSSION—In summary, *Isoetes howellii* has the capacity to fix CO_2 in the dark into malic acid and other acids to a lesser extent. These acids are stored overnight, resulting in an increase of 75–200 μeq titratable acidity g^{-1}FW between 6:00 p.m. and 6:00 a.m. Daytime deacidification is apparently accompanied by C_3 photosynthesis.

Some of these traits have been found in certain other aquatic plants. Dark (or in these cases "light-independent") CO_2-fixation via β-carboxylation has been found in a variety of marine and freshwater plants. In macrophytic marine algae, this type of CO_2-fixation appears to be widespread being best developed in the brown algae (Phaeophyta). In marine algae, CO_2-fixation in the dark differs biochemically from CAM in several ways: PEP carboxy*kinase* is the primary carboxylating enzyme; the dark fixation products are generally amino acids which do not accumulate overnight, and the same CO_2-fixation pathway also operates in the light (Craigie, 1963; Akagawa, Ikawa and Hisizawa, 1972; Willenbrink et al., 1979). Recent work by Holaday and Bowes (1980) indicates that during the summer the freshwater *Hydrilla verticillata* fixes CO_2 in the dark (apparently catalyzed by PEP carboxylase). The same pathway operates in the light, concomitant with RUDP-carboxylation of exogenously supplied CO_2. In the light, malate and aspartate are the main initial β-carboxylation products. At night, most of the dark-fixed carbon is consumed in dark respiration, although some accumulates since titratable acidity increases ca. 20 μeq g^{-1}FW overnight.

The data for *Isoetes howellii* appear to meet the essential metabolic criteria suggested by Kluge and Ting (1978) as defining CAM. Perhaps the major physiological difference between the "prototype" CAM plant and *Isoetes howellii* is that in most terrestrial CAM plants, stomates open at night and close during the day so the CO_2 uptake is restricted to nighttime, whereas *I. howellii* stomata are apparently nonfunctional (Sculthorpe, 1967; pers. observ.) and these plants have a capacity for significant daytime CO_2 uptake.

If future experiments confirm that *Isoetes* has "true" CAM, it would represent not only an example of an aquatic CAM plant, but one in which CAM was selected for a function other than conserving water. *Isoetes* also would represent an example of a CAM plant which is not succulent, either in the "typical sense," or by Kluge and Ting's (1978) index of "mesophyll succulence" (Sm = g water/mg chlorophyll = 0.91, N = 8, for *I. howellii*). Finally, *Isoetes* may represent the most primitive type of CAM plant and the only one with microphyllous leaves; although epiphytic ferns with succulent leaves are reported to be CAM (Wong and Hew, 1976) and the Pteropsida extend back to the Devonian, all members of this subdivision possess the macrophyllous type leaf typical of "higher plants."

The possession of a CAM-like diurnal acid metabolism in *Isoetes howellii* raises an important functional question. In terrestrial CAM plants, daytime decarboxylation generates an internal CO_2 source. This was undoubtedly selected-for because it allows the plant to carry on photosynthesis with stomates closed, thereby conserving water. However, there may be other reasons why generation of an internal CO_2 source during the day would be selected. In some aquatics, photosynthesis may be limited by CO_2 depletion in and around the plant. This may be so even though the total inorganic carbon pool can be greater than in the ambient air. This is related to two factors: the diffusive resistance to CO_2 transfer in water is quite high, and not all of the carbon is in an available form.

The availability of inorganic carbon is dependent on pH and species-specific differences. Below pH 6, free CO_2 predominates, but is replaced by HCO_3^- and CO_3^{2-} at higher pH. According to Raven (1970), free CO_2 is the "preferred" substrate for photosynthesis by terrestrial and aquatic plants. During the day, as free CO_2 is depleted, the pH of unbuffered pools rises. Above pH 8, free-CO_2 is in very low concentration and HCO_3^- is the dominant inorganic carbon source. Some aquatic plants are capable of assimilating HCO_3^- but many are not.

A reasonable working hypothesis is that the primary selective advantage of CAM in *I. howellii* is that this pathway provides an internal CO_2 source during the day when CO_2 becomes limiting to C_3 photosynthesis. The pools which *Isoetes* typically inhabits are heavily vegetated, very shallow and clear, and light intensity and temperature are very high. As a result, photosynthetic depletion of CO_2 may be very rapid as evidenced, by the fact that on warm days oxygen concentration will reach over 200% saturation and free carbon dioxide levels approach zero by noon in these pools. Concomitantly, over 80% of the deacidification will have taken place by noon under such conditions (Keeley, unpubl. data).

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NOTE ADDED IN PROOF: Recent gas exchange studies in conjunction with Dr. George Bowes (using techniques described in Holaday and Bowes, 1980) indicate that *I. howellii* is capable of substantial *net* CO_2 uptake in the dark.