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C_4 Acid Metabolism and Dark CO_2 Fixation in a Submersed Aquatic Macrophyte (*Hydrilla verticillata*)[1]

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ABSTRACT

The CO_2 compensation point of the submersed aquatic macrophyte *Hydrilla verticillata* varied from high (above 50 microliters per liter) to low (10 to 25 microliters per liter) values, depending on the growth conditions. Plants from the lake in winter or after incubation in an 11 C/9-hour photoperiod had high values, whereas summer plants or those incubated in a 27 C/14-hour photoperiod had low values. The plants with low CO_2 compensation points exhibited dark $^{14}CO_2$ fixation rates that were up to 30% of the light fixation rates. This fixation reduced respiratory CO_2 loss, but did not result in a net uptake of CO_2 at night. The low compensation point plants also showed diurnal fluctuations in titratable acid, such as occur in Crassulacean acid metabolism plants. However, dark fixation and diurnal acid fluctuations were negligible in *Hydrilla* plants with high CO_2 compensation points.

Exposure of the low compensation point plants to 20 micromolar $^{14}CO_2$ resulted in 60% of the ^{14}C being incorporated into malate and aspartate, with only 16% in sugar phosphates. At a high CO_2 level, the C_4 acid label was decreased. A pulse-chase study indicated that the ^{14}C in malate, but not aspartate, decreased after a long (270-second) chase period; thus, the C_4 acid turnover was much slower than in C_4 plants.

Phosphoenolpyruvate carboxylase activity was high (330 micromoles per milligram chlorophyll per hour), as compared to ribulose bisphosphate carboxylase (20 to 25), in the plants with low compensation points. These plants also had a pyruvate, Pi dikinase activity in the leaves of 41 micromoles per milligram chlorophyll per hour, which suggests they are not C_3 plants. NAD- and $NADP^+$-malate dehydrogenase activities were 6136 and 24.5 micromoles per milligram chlorophyll per hour, respectively. Of the three decarboxylating enzymes assayed, the activities of NAD- and $NADP^+$-malic enzyme were 104.2 and 23.7 micromoles per milligram chlorophyll per hour, while phosphoenolpyruvate carboxykinase was only 0.2.

Low compensation point *Hydrilla* plants fix some CO_2 into C_4 acids, which can be decarboxylated for later refixation, presumably into the Calvin cycle. Refixation would be advantageous in summer lake environments where the CO_2 levels are high at night but low during the day. *Hydrilla* does not fit any of the present photosynthetic categories, and may have to be placed into a new group, together with other submersed aquatic macrophytes that have environmentally variable CO_2 compensation points.

The CO_2 compensation point has been used as one indicator of the photosynthetic category to which a plant belongs. At 21% O_2, C_3 plants have Γ[4] values of 35 to 70 μl CO_2/l, reflecting their dependence on the O_2-sensitive RuBP carboxylase-oxygenase for the initial carboxylation (4), while C_4 plants have values close to zero, as they utilize the O_2-insensitive PEP carboxylase for the initial carboxylation (3). CAM plants exhibit low Γ values at night when fixation is via PEP carboxylase, and higher values during the day (22). For submersed aquatic macrophytes, the reported values for Γ range from 0 to 85 μl CO_2/l (5, 14, 18, 25, 26). Recent studies with *Hydrilla* and other submersed aquatic macrophytes indicate that their Γ values can be substantially modified by their growth conditions (2). The variability seems to be partially correlated with changes in the activity of PEP carboxylase (2), which fixes CO_2 into C_4 acids.

In C_4 plants, the C_4 acids malate and aspartate, are the major initial photosynthetic products. They function as photosynthetic intermediates (7, 15), being rapidly decarboxylated to release CO_2 for refixation by RuBP carboxylase-oxygenase. Plants operating in CAM also form C_4 acids, mainly malate, as photosynthetic intermediates. However, the major synthesis of C_4 acids occurs at night, with decarboxylation and sugar-P synthesis predominating during the day (20). This time lag between malate formation and decarboxylation results in high acid levels at night that decline during the day. In contrast to C_4 and CAM, C_3 plants fix CO_2 directly into P-glycerate of the Calvin cycle.

Studies of the photosynthetic products in submersed aquatic macrophytes have produced apparently conflicting results. *Myriophyllum* formed principally 3-P-glycerate, glycolate, and glucose-6-P (25), and pulse-chase data for *Egeria* followed the pattern for a C_3 plant (6). Although 3-P-glycerate was the major first product in *Egeria* and *Lagarosiphon*, C_4 acids accounted for up to 30% of the ^{14}C incorporated (5, 6). In *Elodea*, a close relative of *Egeria*, over 45% of the ^{14}C was also recovered in C_4 acids, with only 25% in 3-P-glycerate (8); the per cent label in the C_4 acids did not diminish with increasing chase periods. These relatively high levels of C_4 acids are atypical of C_3 plants. Despite the ability to form substantial amounts of C_4 acids, there are no reports as to the dark CO_2 fixation capacity of submersed aquatic macrophytes, or of their diurnal acidity levels.

Three enzymes have been reported capable of decarboxylating C_4 acids in C_4 and CAM plants: PEP carboxykinase, NAD-malic enzyme, and $NADP^+$-malic enzyme (10, 20). There are no reports as to the activity of these enzymes in submersed aquatic macrophytes. Similarly, malate dehydrogenase is required for the interconversion of OAA and malate, and pyruvate, Pi dikinase for the formation of PEP in C_4 plants (12, 13). The presence of these enzymes in submersed aquatic macrophytes has not been reported.

This paper examines several aspects of C_4 acid metabolism in the submersed aquatic angiosperm *Hydrilla verticillata*, and the manner in which this metabolism changes in response to induced high and low Γ values.

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[4] Abbreviations: Γ: CO_2 compensation point; RuBP: ribulose 1,5-bisphosphate; PEP: phosphoenolpyruvate; OAA: oxaloacetate.

MATERIALS AND METHODS

Plant. *H. verticillata* Royle was collected from Orange Lake, Fla. Apical sections 10 cm in length were cut under water and either utilized on the day of collection or after being incubated for 1 week in growth chambers. The plants were incubated in 200-ml gas washing bottles containing a 5% (v/v) Hoagland solution at pH 5.5 with a 14 h, 27 C day/20 C night to induce low Γ values below 25 μl CO_2/l, or a 9 h, 11 C day/6 C night to induce high Γ values above 60 μl CO_2/l (2). The Hoagland solution was changed daily, and all plants were washed before use to remove epiphytes. Leaves of *Sorghum bicolor* (L.) Moench. and *Panicum maximum* Jacq. were obtained from greenhouse grown plants; spinach (*Spinacia oleracea* L.) was field-grown.

IR Gas Analysis Measurements. Respiration rates and Γ values were determined at 25 C in the system described previously (26). Respiration rates were determined in the dark from the time required for the plant material in the closed system to increase the CO_2 level of the circulating gas mixture from 335 to 340 μl CO_2/l (gas phase).

Titratable Acidity Determinations. Apical sections of *Hydrilla* (approximately 1 g fresh weight) were obtained from Orange Lake or the growth chambers at 06:00 h and again at 15:00 h and transported on ice to the laboratory for analysis of total acidity by the method of Moradshahi *et al.* (19).

$^{14}CO_2$ Fixation Experiments. $^{14}CO_2$ fixation rates in the light and dark were determined for *Hydrilla* at 25 C in stoppered, 200-ml gas washing bottles containing a 5% (v/v) Hoagland solution at pH 5.5 and 5 μCi of 20 μM $NaH^{14}CO_3$. Prior to $NaH^{14}CO_3$ addition, the Hoagland solution was sparged with CO_2-free air to remove air-CO_2 and establish air-equilibrium O_2 levels. Apical sections of *Hydrilla* were equilibrated for 2 h in an aerated 5% (v/v) Hoagland solution, either at a saturating quantum flux density (800 μE/m^2·s, 400–700 nm) or in the dark, before transferring them rapidly to the $^{14}CO_2$ solutions. They were then allowed to fix $^{14}CO_2$ for periods ranging from 5 to 10 min, during which time the $^{14}CO_2$ concentration of the solution did not decrease by more than 5%. The $^{14}CO_2$ uptake was stopped by plunging the plants into liquid N_2. They were ground in liquid N_2 and then brought to 100 ml with 80% (v/v) acetone. Aliquots were taken for Chl determinations (1). Further aliquots were taken, acidified with 6 M HCl saturated with 2,4-dinitrophenylhydrazine, dried in an air stream to remove unfixed $^{14}CO_2$, and their ^{14}C content determined by liquid scintillation spectrometry. Nighttime CO_2 fixation determinations (in the dark and light) were performed 2 h after the chamber-grown plants entered their dark period, or 2 h after sunset for lake-grown plants.

Time Course and Pulse-Chase Experiments. Apical sections of *Hydrilla* exhibiting low Γ values were equilibrated at 25 C for 1 h in a 5% (v/v) Hoagland solution with a saturating quantum flux density and air levels of CO_2 and O_2. For time course measurements, the plants were transferred rapidly to 5% (v/v) Hoagland solution containing either 20 or 250 μM $NaH^{14}CO_3$ (50 μCi) for periods ranging from 10 to 120 s. The Hoagland solution was flushed with CO_2-free air before $NaH^{14}CO_3$ addition. Fixation was terminated by plunging the plants into liquid N_2. For pulse-chase experiments, the equilibrated plants were "pulsed" for 30 s in Hoagland solution containing 20 μM $NaH^{14}CO_3$ (50 μCi), quickly rinsed in Hoagland solution containing unlabeled 20 μM $NaHCO_3$, and then held for various times in a similarly unlabeled solution before being plunged into liquid N_2. All operations were performed in the light so that the time spent in the rinse and final solutions constituted the "chase" period.

Labeled compounds were extracted by a procedure modified from that of Chen *et al.* (7). The plants were ground in liquid N_2. Each sample was brought to 25 ml with 85% (v/v) ethanol containing 0.02% (w/v) phenylhydrazine and aliquots were taken to determine by liquid scintillation spectrometry the total amount of $^{14}CO_2$ fixed. The ^{14}C content of the insoluble, water-soluble, and ethanol-soluble fractions was also determined. Two-dimensional paper chromatography was used to separate the water-soluble compounds (23), and their positions were visualized by autoradiography. The identity of radioactive spots was ascertained by chromatography of authentic labeled compounds.

When authentic ^{14}C-labeled malate, aspartate, 3-P-glycerate, fructose 1, 6-bisP, or alanine was added to the plant extracts, recovery of the label was in excess of 99%; but the per cent recovery of the total ^{14}C fixed by the plants was consistently lower. This suggests that although the per cent recovery of known compounds was high, one or more unknown labeled compounds (possibly OAA) were lost during the extraction and identification process, despite the precautions taken.

Enzyme Assays. The assays were performed on extracts prepared by grinding 1.0 g of *Hydrilla* leaves, or 0.2 g of the upper leaves of *Sorghum*, *Panicum*, or spinach in a TenBroeck homogenizer in a solution at pH 7.0 containing 25 mM Hepes-NaOH, 1 mM $MgCl_2$, 5 mM DTT, and 1% (w/v) PVP-40 at 4 C. Aliquots were taken for Chl determination (1), and the homogenates were either filtered through two layers of Miracloth or centrifuged at 10,000*g* for 5 min. For measurements of pyruvate, Pi dikinase activity the leaves were harvested during the light period, ground under a N_2 atmosphere, and the supernatant was activated at 30 C for 15 min prior to assay.

Pyruvate, Pi dikinase activity was assayed as described by Hatch and Slack (13), except the pyruvate- and Pi-dependent formation of PEP was determined from the amount of $H^{14}CO_3^-$ fixed into OAA. Purified PEP carboxylase (0.2 units/assay) was purchased from Sigma Chemical Co. The reaction was halted by the addition of 0.1 ml of 6 M HCl saturated with 2,4-dinitrophenylhydrazine, and the solutions were dried at 35 C in an air stream before determining the radioactivity by liquid scintillation spectrometry. PEP carboxylase activity was determined according to the procedure of Van *et al.* (26). PEP carboxykinase activity was analyzed using the ATP-dependent exchange reaction between OAA and PEP (9). Malate dehydrogenase was assayed spectrophotometrically at 340 nm by measuring the decrease in *A* as NADH or NADPH was oxidized (16). Malic enzyme activity was also determined spectrophotometrically at 340 nm from the increase in *A* and NAD or $NADP^+$ was reduced, after equilibration of any malate dehydrogenase activity (10, 11). All reported enzyme activity values were derived from at least three separate determinations.

RESULTS

As we reported previously (2), the Γ values of *Hydrilla* plants varied with the environmental growth conditions (Table I). Values of Γ were lowest when the plants were exposed to a long photoperiod and relatively high temperatures, such as would be experienced in the summer. Under these conditions, low Γ values approaching those of terrestrial C_4 plants could be induced (see

Table I. *Light and Dark $^{14}CO_2$ Fixation Rates Measured at 21:00 h with Hydrilla from Various Growth Conditions*

	Growth Conditions	Γ	Dark Fixation	Light Fixation
		μl CO_2/l	*μmol CO_2/mg Chl·h*	
Growth chamber	27 C/14-h photoperiod	25	0.42 ± 0.11[a]	2.65 ± 0.41
Lake	Summer/dense vegetation	25	0.73 ± 0.04	
Lake	Summer/open water	30	0.30 ± 0.08	2.52 ± 0.33
Lake	Winter/open water	87	0.08 ± 0.01	2.12 ± 0.18

[a] Mean of two replicates ± SD.

also Table III). In contrast, plants collected from the lake in the winter had the highest Γ value (Table I).

This variability in Γ was correlated with the ability of the plants to incorporate $^{14}CO_2$ in the dark. Plants with low Γ values, collected in the summer from the lake, exhibited the highest dark CO_2 fixation rates (Table I), which were up to 30% of the light fixation rates. Plants with low Γ values induced by incubation in the growth chamber also had high dark fixation rates. However, winter-grown plants from the lake, with high Γ values, exhibited dark fixation rates that were only about 4% of the light fixation rates (Table I).

IR gas analysis measurements indicated that the low Γ values in the light remained unchanged, irrespective of whether they were determined during the day or the night period (Table II). In contrast, CO_2 evolution in the dark (dark respiration) was 40% less during the night than during the day (Table II). Dark respiration rates were relatively high when compared with the photosynthetic rates, as has been observed previously for submersed aquatic angiosperms (26). Although these plants were capable of substantial CO_2 fixation in the dark, it did not result in net CO_2 uptake at night (Table II).

Hydrilla plants with low Γ values exhibited diurnal fluctuations in titratable acidity (Table III), irrespective of whether the low Γ values were induced naturally in the lake or artificially by incubation in a growth chamber. Acid levels in these plants at the end of the night period (06:00 h) were substantially higher than in the afternoon (15:00 h). The plants with high Γ values did not exhibit fluctuations in titratable acidity, although the acidity levels were as high as the nighttime levels of plants with low Γ values (Table III).

Table IV shows a time course study of photosynthetic $^{14}CO_2$ incorporation for *Hydrilla* plants with low Γ values. The CO_2 concentration employed (20 μM) was close to the levels found in Florida lakes during the early part of the day (26). After a 10-s exposure to $^{14}CO_2$, all of the ^{14}C in the water-soluble products was in sugar-P, malate, and aspartate; 60% was in the C_4 acids, malate and aspartate, and only 9% was in 3-P-glycerate. With longer exposure times the per cent label in malate and 3-P-glycerate declined, while total sugar-P and aspartate remained relatively unchanged. The per cent label in the unidentified, water-soluble, ethanol-soluble, and insoluble fractions increased with longer incorporation times.

When the CO_2 concentration was raised from 20 to 250 μM, the $^{14}CO_2$ fixation rate increased from 4.0 to 22.3 μmol/mg Chl·h (Tables IV and V), and the distribution of label was changed. After a 10-s exposure, the percentage of ^{14}C in the C_4 acids was less than half of that found at 20 μM while the label in sugar-P was increased (Table V). With increased exposure times the percentage of label in malate remained unchanged, while that in aspartate increased. Due to this increase in aspartate labeling, after a 120-s exposure the total per cent label in C_4 acids was similar for both CO_2 concentrations employed. The label in the ethanol-soluble and insoluble fractions was alike at both CO_2 concentrations.

In a pulse-chase experiment, to examine the fate of the C_4 acids in *Hydrilla* plants at 20 μM CO_2, the label in malate was found to increase with chase times up to 40 s (total time 70 s), but then decreased after a long chase period of 270 s (Fig. 1). The continued increase in malate label during the initial chase period was probably due to difficulties in rapidly washing off the $^{14}CO_2$. The label in aspartate increased throughout the pulse and chase periods (Fig. 1). The initial label in 3-P-glycerate and other sugar-P showed a steady decline with increasing chase periods (data not shown).

In view of the apparent predominance of acid production in

Table II. *Gas Exchange Measurements of the CO_2 Compensation Point in the Light and the CO_2 Evolution Rate in the Dark for Hydrilla at 14:00 and 20:00 h*

The low Γ values were induced by incubation of the plants for 1 week in a growth chamber with a 14 h, 27-C day/20-C night.

Time Measured	Γ	CO_2 Evolution in the Dark
h	*μl CO_2/l*	*μmol CO_2/mg Chl·h*
14:00	25	1.69 ± 0.24[a]
20:00	27	1.02 ± 0.15

[a] Mean of three replicates ± SD.

Table III. *Diurnal (Early Morning and Late Afternoon) Levels of Titratable Acidity and CO_2 Compensation Points for Hydrilla as a Function of Various Growth Conditions*

	Growth Conditions	Titratable Acidity 06:00 h	Titratable Acidity 15:00 h	Γ
		μeq acid/g fresh wt	*μeq acid/g fresh wt*	*μl CO_2/l*
Lake	Summer/dense vegetation	51.0 ± 13.0[a]	29.7 ± 5.8	23
Lake	Winter/ open water	67.1 ± 1.2	65.5 ± 0.7	87
Growth chamber	27 C/14-h photoperiod	52.6 ± 3.6	39.7 ± 2.3	10
Growth chamber	11 C/9-h photoperiod	52.3 ± 2.7	62.0 ± 1.9	54

[a] Mean of three replicates ± SD.

Table IV. *Time Course Showing the Percentage Incorporation of ^{14}C into Photosynthetic Intermediates at 20 μM CO_2 for Hydrilla with a CO_2 Compensation Point of 25 μl/l*

Total dpm fixed at each time was: 10 s: 717,348; 30 s: 1,521,610; 120 s: 8,257,641. Mean photosynthetic rate was 4.0 μmol CO_2/mg Chl·h.

Fraction	^{14}C Fixed 10 s	30 s	120 s
		%	
Water-soluble	75.7	85.7	72.5
Total sugar-P	16.3	13.6	16.2
P-glycerate	8.9	1.8	5.0
Malate	41.4	32.4	25.3
Aspartate	18.1	27.0	21.5
Unidentified	0.0	12.6	9.4
Ethanol-soluble	3.8	5.3	11.2
Insoluble	1.8	1.8	4.3
^{14}C recovered	79.5	91.0	83.7

Table V. *Time Course Showing the Percentage Incorporation of ^{14}C into Photosynthetic Intermediates at 250 μM CO_2 for Hydrilla with a CO_2 Compensation Point of 25 μl/l*

Total dpm fixed at each time was: 10 s: 299,436; 30 s: 1,143,470; 120 s: 2,990,997. Mean photosynthetic rate was 22.3 μmol CO_2/mg Chl·h.

Fraction	^{14}C Fixed 10 s	30 s	120 s
		%	
Water-soluble	49.2	79.0	70.7
Total sugar-P	24.0	36.4	21.1
P-glycerate	9.0	14.3	3.3
Malate	18.6	18.5	18.5
Aspartate	6.6	19.9	26.5
Unidentified	0.0	4.2	3.2
Ethanol-soluble	4.1	5.2	12.0
Insoluble	0.9	2.6	1.8
^{14}C recovered	53.3	84.2	82.7

Hydrilla plants with low Γ values, during both photosynthetic and dark CO_2 fixation, the activities of several enzymes known to be involved in the C_4 acid metabolism of C_4 and CAM plants were determined (Table VI). Data for two terrestrial C_4 plants (*Sorghum* and *Panicum*) and one C_3 plant (spinach) are included in Table VI for comparison. *Hydrilla* with low Γ values exhibited high PEP carboxylase activity (Table VI), although not as high as in the terrestrial C_4 plants studied. The PEP carboxylase activity was much higher than the RuBP carboxylase activity, which was only 20 to 25 μmol CO_2/mg Chl·h in these low Γ *Hydrilla* plants.

In C_4 and certain CAM plants, PEP formation is catalyzed by pyruvate, Pi dikinase (13, 20). Appreciable activity of this enzyme was found in *Hydrilla* and *Sorghum* leaves, although none was found in spinach (Table VI). Malate dehydrogenase activity was also present in *Hydrilla*, especially the NAD-dependent activity which was almost 2-fold greater than that found in *Sorghum*. Of the three decarboxylation enzymes assayed for in *Hydrilla*, the NAD-malic enzyme was the highest (Table VI). $NADP^+$-malic enzyme was also present (Table VI), and was inhibited by 1 mM oxalate (data not shown). This inhibition indicated that it was not just residual NAD-malic enzyme, which is oxalate-insensitive. PEP carboxykinase was virtually undetectable in *Hydrilla*, although it was found in *Panicum*.

DISCUSSION

In low Γ *Hydrilla*, dark CO_2 fixation was equivalent to 30% of the light fixation rate, which is similar to CAM plants (20), but much higher than found with C_3 and C_4 plants, where it is generally less than 1% (17). The dark fixation in CAM plants is a consequence of high PEP carboxylase activity and stomata that open at night (20). *Hydrilla* plants with the highest dark fixation exhibited PEP carboxylase levels that were up to 5-fold greater than in plants with low dark fixation capability (A.S. Holaday, and G. Bowes, unpublished results), which suggests that the dark fixation is a function of PEP carboxylase activity. Furthermore, *Hydrilla* leaves lack stomata, and therefore are not subject to diurnal changes in the stomatal resistance to CO_2 diffusion. Because of high respiratory activity the dark CO_2 fixation in *Hydrilla*

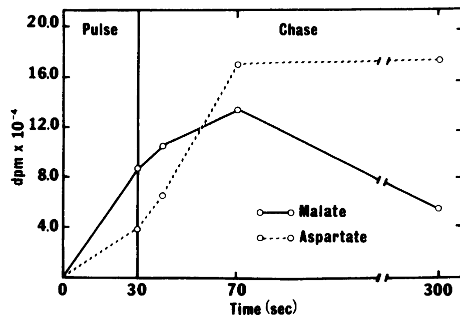

FIG. 1. Pulse-chase experiment showing the incorporation of ^{14}C into malate and aspartate for *Hydrilla* plants with low Γ. Plants were exposed to a 30-s pulse of $NaH^{14}CO_3$ followed by $NaH^{12}CO_3$ for chase periods of up to 270 s.

does not result in net CO_2 fixation at night; rather it leads to a marked reduction in nighttime respiratory CO_2 evolution. The cause of the relatively high respiration in submersed aquatic angiosperms is unknown, but substantial refixation during the night of the respired CO_2 could serve as a carbon conservation measure in lake environments where the daytime CO_2 levels may be severely limiting (26).

Hydrilla plants capable of fixing CO_2 at night also exhibited diurnal changes in titratable acidity, somewhat similar to those found for plants operating in CAM (19). The increased acidity, together with the high PEP carboxylase activity, suggests that C_4 acids were being produced during the night, although whether they were stored as such is unknown. For both high and low Γ plants the nighttime acid pool sizes were similar; the major difference was in the daytime pool sizes. This indicates that the turnover, or utilization rate, during the day was greater for the low Γ than for the high Γ plants.

Considerable C_4 acid synthesis occurred in low Γ *Hydrilla* plants during the day, suggesting that PEP carboxylase is utilized for CO_2 fixation both day and night. The proportion of label entering C_4 acids during the day was typical of that reported for terrestrial C_4 plants (15, 24), and considerably greater than that normally found for C_3 or CAM plants (21, 24). Aspartate was a major component of the acid production in *Hydrilla*.

The labeling pattern in *Hydrilla* was similar to that reported for *Elodea* (8), but other studies of submersed aquatic macrophytes have indicated that sugar-P predominates (5, 6, 25). Several factors could account for the apparent discrepancies. Brown *et al.* (5), for their observation that the ^{14}C fixation data of *Egeria* and *Lagarosiphon* resembled that of C_3 plants, used plants with high Γ values. Our investigations have shown that PEP carboxylase activity decreases with an increase in Γ (2). Browse *et al.* (6) used a high CO_2 concentration, which from the present study should initially decrease the proportion of label entering C_4 acids. Finally, high pH values apparently favor sugar-P labeling (5). Thus, the Γ value of the plant, and the CO_2 and H^+ ion concentrations all may be important determinants of the relative contributions that PEP and RuBP carboxylases make to the total carbon fixation of submersed aquatic macrophytes.

From the *Hydrilla* pulse-chase data, the more rapid labeling of malate than aspartate suggests product-precursor relationships, probably with OAA. Unlike aspartate, some turnover of malate appeared to occur after a long (270-s) chase period. The failure of DeGroote and Kennedy (8) to find malate turnover in *Elodea* may have been due to the short chase period they employed. The lack of a rapid turnover in C_4 acids indicates that C_4 photosynthesis, as such, does not occur in *Hydrilla* or *Elodea*, although under certain conditions substantial C_4 acid metabolism may occur, as evidenced by the labeling data, the level of PEP carboxylase, and by the existence of diurnal acidity changes.

The fate of the C_4 acids is uncertain. It is possible that they function as photosynthetic intermediates, being stored for decarboxylation during the day. *Hydrilla* leaves do possess a layer of large, vacuolated cells, somewhat similar to those in CAM plants, which could be utilized for storage. Upon decarboxylation, the CO_2 could then be refixed by RuBP carboxylase. The resistance

Table VI. *Activities of PEP Carboxylase, Pyruvate, Pi Dikinase, Malate Dehydrogenase, Malic Enzyme, and PEP Carboxykinase in Hydrilla and Three Terrestrial Plants*

Plant Material	PEP Carboxylase	Pyruvate, Pi Dikinase	Malate Dehydrogenase		Malic Enzyme		PEP Carboxykinase
			NAD	$NADP^+$	NAD	$NADP^+$	
				μmol/mg Chl·h			
Hydrilla	330	41.4	6136	24.5	104.2	23.7	0.2
Spinacia (C_3)	76	0					
Sorghum (C_4)	957	75.3	3187	48.2	37.3	475	
Panicum (C_4)	983						81.4

to CO_2 diffusion in an aquatic environment would aid internal refixation. In a heavily vegetated lake environment, with high CO_2 levels in the water at night, and negligible levels during much of the day (26), such a refixation strategy would be advantageous.

Of the three enzymes known to catalyze the decarboxylation of C_4 acids in higher plants, NAD- and possibly $NADP^+$-malic enzymes were found in *Hydrilla* with sufficient activity to utilize the C_4 acids over a period of hours. C_4 NAD-malic enzyme plants may possess a similar enzyme system, but their decarboxylation activity is somewhat higher than that of *Hydrilla* (10, 12). Although CAM plants may exhibit high NAD-malic enzyme activity, they also generally possess high activity of either $NADP^+$-malic enzyme or PEP carboxykinase (20).

NAD-malate dehydrogenase activity in *Hydrilla* was high compared to C_4 plants such as *Sorghum*, but was in the range for C_3, CAM, and NAD-malic enzyme C_4 plants (9, 12). The $NADP^+$-malate dehydrogenase activity of *Hydrilla* was also similar to values reported for C_3 and CAM plants, but was lower than in C_4 plants (12). However, the finding in the present study of pyruvate, Pi dikinase activity in the leaves of *Hydrilla* is an indication that this plant does not belong to the C_3 category.

Superficially, the changes in the photosynthetic carbon metabolism of *Hydrilla* resemble those found in facultative CAM plants (20). In both cases, environmental factors influence the ability of the plants to fix CO_2 at night, and the magnitude of the diurnal titratable acidity change. However, unlike most plants operating in CAM, *Hydrilla* plants capable of dark fixation did not exhibit net CO_2 uptake at night. Also the Γ values measured during the day for *Hydrilla* plants capable of substantial dark fixation were much lower than those reported for plants operating in the CAM mode (20, 22).

This evidence may require that *Hydrilla*, and other submersed aquatic macrophytes which exhibit environmentally induced variations in their Γ values (2), be classified in a new photosynthetic category.

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