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# Two Photosynthetic Mechanisms Mediating the Low Photorespiratory State in Submersed Aquatic Angiosperms[1]

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## ABSTRACT

**The submersed angiosperms *Myriophyllum spicatum* L. and *Hydrilla verticillata* (L.f.) Royal exhibited different photosynthetic pulse-chase labeling patterns. In *Hydrilla*, over 50% of the $^{14}C$ was initially in malate and aspartate, but the fate of the malate depended upon the photorespiratory state of the plant. In low photorespiration *Hydrilla*, malate label decreased rapidly during an unlabeled chase, whereas labeling of sucrose and starch increased. In contrast, for high photorespiration *Hydrilla*, malate labeling continued to increase during a 2-hour chase. Thus, malate formation occurs in both photorespiratory states, but reduced photorespiration results when this malate is utilized in the light. Unlike *Hydrilla*, in low photorespiration *Myriophyllum*, $^{14}C$ incorporation was via the Calvin cycle, and less than 10% was in $C_4$ acids.**

**Ethoxyzolamide, a carbonic anhydrase inhibitor and a repressor of the low photorespiratory state, increased the label in glycolate, glycine, and serine of *Myriophyllum*. Isonicotinic acid hydrazide increased glycine labeling of low photorespiration *Myriophyllum* from 14 to 25%, and from 12 to 48% with high photorespiration plants. Similar trends were observed with *Hydrilla*. Increasing $O_2$ increased the per cent [$^{14}C$]glycine and the $O_2$ inhibition of photosynthesis in *Myriophyllum*. In low photorespiration *Myriophyllum*, glycine labeling and $O_2$ inhibition of photosynthesis were independent of the $CO_2$ level, but in high photorespiration plants the $O_2$ inhibition was competitively decreased by $CO_2$. Thus, in low but not high photorespiration plants, glycine labeling and $O_2$ inhibition appeared to be uncoupled from the external $[O_2]/[CO_2]$ ratio.**

**These data indicate that the low photorespiratory states of *Hydrilla* and *Myriophyllum* are mediated by different mechanisms, the former being $C_4$-like, while the latter resembles that of low $CO_2$-grown algae. Both may require carbonic anhydrase to enhance the use of inorganic carbon for reducing photorespiration.**

Terrestrial $C_3$ and $C_4$ plants can be distinguished on the biochemical basis of the first products of carbon fixation. However, they concomitantly exhibit characteristic physiological and ecological differences, especially in regard to gas exchange (10). Consequently, the terms $C_3$ and $C_4$ take on a much wider meaning when they are used to describe the complete syndrome of characteristics associated with each group. This wider interpretation applies to the term $C_3$–$C_4$ intermediates, in describing terrestrial plants which exhibit gas exchange characteristics, anatomical features, and, for some species, levels of $C_4$ acid metabolism, which are intermediate between $C_3$ and $C_4$ plants (14). In the same vein, the term Crassulacean acid metabolism refers to a cadre of characteristics, and not just to the biochemical mechanism or family involved (19).

Aquatic autotrophs pose a problem in regard to categorization. Certain unicellular green algae (3, 4, 25, 26) and cyanobacteria (3) exhibit changeable photosynthetic and photorespiratory characteristics depending upon the $CO_2$ concentration at which they are grown. Based on the first product of carbon fixation, they are 'biochemically $C_3$' plants (4); however, this designation becomes confusing for low $CO_2$-grown algae which physiologically are $C_4$-like. These algae and cyanobacteria also differ from $C_3$–$C_4$ intermediates in that they exhibit a continuum of photorespiratory states (3) and not a fixed intermediate level.

Submersed aquatic macrophytes of freshwater also exhibit a continuum of photorespiratory states, ranging from high to low (7, 15, 21). Their status is further complicated by evidence that in some aquatic plants $C_4$ acids can be major photosynthetic products (9, 11, 13, 17), even in those with photorespiratory activity (9). In addition, features such as dark $^{14}C$ fixation, net fixation of $CO_2$ in the dark, and diurnal fluctuations in the level of titratable acidity, which are generally associated with CAM plants (19), have been observed in certain submersed plants (5, 9, 13, 15, 17, 18). Thus, photosynthetic carbon metabolism in submersed aquatic macrophytes can involve a combination of features, each generally thought to be characteristic of a particular photosynthetic category.

Part of the difficulty results from our lack of understanding as to how the pathways for carbon assimilation in submersed plants relate to the level and variability of photorespiratory activity (9, 13). A labeling study with *Myriophyllum* in an undetermined photorespiratory state, indicated that carbon assimilation was via the Calvin cycle (27). Reportedly low levels of PEP[4] carboxylase activity, even with the plant in the low photorespiratory state, support this observation (21, 22). In contrast, for *Hydrilla*, $C_4$ acids were major early products of photosynthesis and the induction of the low photorespiratory state was associated with an increase in the activities of enzymes involved in $C_4$ acid metabolism (13, 21). $C_4$ acids have been reported in other submersed angiosperms (9, 11, 12), but their photosynthetic role is unclear. Pulse-chase labeling experiments have not resolved this question, as label in malate was reported either not to turn over (11), or to turn over at a low rate (9, 13).

To clarify some of these problems, the relationship between $C_4$ acid metabolism and the variable photorespiratory states in

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[4] Abbreviations: PEP, phosphoenolpyruvate; DNPH, 2,4-dinitrophenylhydrazine; INH, isonicotinic acid hydrazide; PR, photorespiration; RuBP, ribulose 1,5-bisphosphate.

*Hydrilla* was examined, especially with regard to the fate of malate produced in photosynthesis. For comparison, the photosynthetic products of *Myriophyllum* were investigated. Because a low photorespiratory state also is inducible in *Myriophyllum*, an attempt was made to elucidate the mechanism(s) responsible for this state by examining the effects of $O_2$, $CO_2$, ethoxyzolamide, and INH on the gas exchange characteristics, and carbon flux through the photorespiratory pathway. Ethoxyzolamide, an inhibitor of carbonic anhydrase, represses the low photorespiratory state of *Myriophyllum* and *Hydrilla* (23). INH inhibits serine hydroxymethyltransferase activity, and has been used to block carbon flow through the photorespiratory pathway (24).

## MATERIALS AND METHODS

**Plant Material.** *Myriophyllum spicatum* L. and *Hydrilla verticillata* (L. f.) Royal were collected and incubated as described previously (21).

**Infrared Gas Analysis.** Net photosynthetic rates were determiend by measuring $CO_2$ uptake in a closed system (22). $CO_2$ compensation point values were used to verify the photorespiratory state of each plant used in an experiment, and were determined as described by Van *et al.* (30). The $CO_2$ compensation point has been shown to be a reliable indicator of the photorespiratory state (21). All gas exchange measurements were performed at 30°C using a saturating quantum irradiance of 1000 $\mu$mol/m$^2$·s (400–700 nm). Gas exchange data represent mean values from at least three separate determinations.

**Initial Product and Pulse-Chase Experiments.** Carbon fixation experiments were performed at 30°C in the light under a saturating quantum irradiance of 900 $\mu$mol/m$^2$·s (400–700 nm). After a 1 to 2-h equilibration period, apical segments of *Myriophyllum* and *Hydrilla*, 5 to 10 cm in length, were transferred from an aerated (317 $\mu$l $CO_2$/l and 21% $O_2$, gas phase) solution of 10 mM Mes-NaOH and 5% (v/v) Hoagland solution at pH 5.5 (Mes-Hoagland solution) and pulsed for 20 s in 200 ml of Mes-Hoagland solution containing 20 $\mu$M $NaH^{14}CO_3$ (50 $\mu$Ci/$\mu$mol). Prior to the addition of $H^{14}CO_3^-$, the pulse solution was purged for 1 h with $CO_2$-free air. In pulse-chase experiments, the labeled plants were transferred to a second aerating solution to complete the duration of the chase. Aeration established an unlabeled free $CO_2$ concentration of 9.0 $\mu$M for the chase portion of the experiment. Carbon fixation was stopped by rapidly plunging the plants into liquid $N_2$.

Experiments involving ethoxyzolamide employed the above procedures except that ethoxyzolamide at 100 $\mu$M was included in all solutions, and the equilibration period was extended to 4 h (23).

**Glycine Accumulation.** Carbon fixation experiments were performed in a shaking water-bath using detached leaves of *Myriophyllum* and *Hydrilla.* The light and temperature conditions were as described above. Entire apical segments were incubated at 25°C in the dark for 1 h in Mes-Hoagland solution containing various INH concentrations. After 1 h, two leaves (approximately 50 $\mu$g Chl) were detached and added to 25-ml reaction flasks with 10 ml of the INH-containing solution, which had been previously purged for 20 min with a $CO_2$-free $N_2/O_2$ mixture. Purging was continued for 7 min in the light to equilibrate the leaves, after which time the flasks were rapidly sealed and fixation initiated by the addition of 0.1 ml $NaH^{14}CO_3$ (50 $\mu$Ci/$\mu$mol) through a serum cap. After 15 min, carbon fixation was stopped by rapidly plunging the leaves into liquid $N_2$. Experiments were run in duplicate and, for each experiment, the pooled contents from two replicate vials were analyzed.

**Extraction of Labeled Compounds.** The plant material frozen in liquid $N_2$ was ground in a Ten Broeck homogenizer containing 80% (v/v) acetone at 4°C. The resulting homogenate was quantitatively transferred to a 15-ml centrifuge tube and aliquots were taken to determine Chl content (1) and total $^{14}C$ fixed. For the latter, aliquots were added to scintillation vials containing 0.5 ml $H_2O$ acidified with 0.1 ml of a 6 N HCl solution saturated with DNPH. Triplicate determinations for each sample were taken to dryness and $^{14}C$ dpm were determined by liquid scintillation spectrometry. Eighty-five per cent (v/v) ethanol containing 0.12% (w/v) DNPH was added to the remainder of the homogenate and the resulting mixture was warmed to room temperature. After 1 h, the solution was centrifuged for 20 min at 10,000*g* and the pellet was extracted sequentially with boiling solutions of ethanol (85, 40 and 20% [v/v]), distilled $H_2O$ (twice), and 0.5 N formic acid, each for 10 min. The supernatants from each extraction were pooled and taken to dryness overnight at 40°C under a stream of air. The amount of $^{14}C$ label remaining in the pellet represented the insoluble fraction. The supernatant fraction was reconstituted with $H_2O$ and then extracted twice with chloroform to remove lipids and unreacted DNPH, and finally lyophilized. The $^{14}C$ in the chloroform fraction was generally less than 1% of the total $^{14}C$ fixed. Determinations of radioactivity in the chloroform, insoluble, and supernatant fractions indicated that greater than 93% of the total $^{14}C$ fixed was recovered at this stage.

**Separation of Labeled Compounds.** Following the reconstitution of the lyophilate in $H_2O$, the extract was quantitatively fractionated by ion-exchange chromatography (2). Basic compounds were eluted from an 8.0 × 60.0-mm column of Dowex 50 X 8–400 with 2 N $NH_4OH$. Nonbasic compounds were separated into neutral, acid 1, and acid 2 fractions by chromatography on Dowex 1 × 8–400, eluted with $H_2O$, 3 N HCOOH, and 4 N HCl, respectively. These elution conditions, as confirmed by the separation of authentically labeled standards ([$^3$H]glucose, [$^{14}$C]malate, [$^{14}$C]P-glycerate, [$^{14}$C]fructose-1,6-bisP), provided complete separation of malate from P-glycerate. The $^{14}C$ dpm were determined for each fraction and recovery of $^{14}C$ from the ion-exchange columns was nearly 100% as has been reported by other workers (2). Each fraction from the columns was lyophilized and then reconstituted by the addition of 100 $\mu$l of 10% (v/v) ethanol, with the exception of the neutral fraction to which 100 $\mu$l of $H_2O$ was added.

**Thin Layer Chromatography.** Individual compounds in each ion-exchange fraction were separated by one-dimensional chromatography on plastic backed thin-layer plates (MN-Cellulose 300, Brinkmann Instruments). The plates were developed twice in the appropriate solvent system after overspotting five 2-$\mu$l aliquots of each sample using a capillary pipette. Aliquots of each sample were also added directly to scintillation vials with the 2-$\mu$l capillary pipette to determine the total $^{14}C$ added to the plate prior to separation. Recovery of $^{14}C$ from the thin-layer plates, after separation, exceeded 95%. In the basic fraction, amino acids were separated in 1-butanol/acetone/water/diethylamine (20:20:10:3, v/v). Compounds in the acid 1 fraction were separated in ethanol/$NH_4OH$/water (6:1:1.6, v/v, equilibrated for 24 h) and in *sec*-butanol/HCOOH/water (6:1:2, v/v). This latter solvent system was also used for the acid 2 fraction. The neutral fraction was separated by chromatography in 1-butanol/acetic acid/water (12:3:5, v/v). Individual compounds were localized by co-chromatography with authentic standards, and areas corresponding to these markers were cut out and their $^{14}C$ dpm determined. Authentic amino acids, chromatographed in adjacent lanes, were visualized by spraying these marker lanes with ninhydrin. Cold carrier standards were co-chromatographed with the acid 1 fraction and visualized with bromocresol purple and $NH_4OH$. In a similar manner, phosphorylated compounds in the acid 2 fraction were identified using ammonium molybdate.

To check the accuracy of the procedures outlined previously, in some experiments individual compounds were also separated

by two-dimensional TLC of samples taken prior to ion-exchange chromatography. The results were in agreement with those from the procedures outlined above with regard to the identity of, and per cent $^{14}C$ incorporated in, individual compounds. Solvent systems used were *sec*-butanol/HCOOH/water (6:1:2, v/v), ethanol/$NH_4OH$/water 6:1:1.6, v/v), and the phenol/water and butanol/propionic acid/water system of Pratt and Rand (20).

## RESULTS

For *Hydrilla* in both the low and high photorespiratory states, greater than 50% of the label that was incorporated after 20-s photosynthesis in 20 $\mu$M $H^{14}CO_3^-$ was recovered in the $C_4$ acids malate and aspartate (Fig. 1). During the ensuing chase period in unlabeled $CO_2$, the low photorespiration *Hydrilla* plants showed a rapid decline in the per cent $^{14}C$ in both malate and aspartate, after a 1-min lag period (Fig. 1). This decline continued for 60 min. In contrast, in the high photorespiration plants, the malate did not decline during the chase, but instead exhibited an increase in per cent $^{14}C$ incorporation (Fig. 1). Unlike malate, the aspartate labeling of high photorespiration plants declined steadily, dropping from 28 to 5% during the 2-h chase (Fig. 1). This decline in aspartate coincided with the increase in malate labeling (Fig. 1), and also with increases in the amino acids glutamate and alanine (data not shown).

The data in Figure 2 demonstrate that, for the low photorespiration *Hydrilla* plants, the decline in malate and aspartate labeling during the chase period was relatively rapid, with a 50% decrease occurring within 180 s. During this time period, there was a steady and concomitant increase in $^{14}C$ entering the neutral and insoluble fractions, composed largely of sucrose and starch, respectively (Fig. 2). Glycine and serine labeling also rose during the chase period. Concomitant with the rise in sucrose and starch, the per cent label in total phosphorylated compounds showed a slight tendency to decline from an initial value of 32% (Fig. 2).

Figure 3 shows in more detail the labeling patterns for *Hydrilla* during the extended 2-h chase period. Both high and low photorespiration plants initially exhibited similar percentages of $^{14}C$ incorporation into Calvin cycle intermediates. During the chase period, for both photorespiration types, the phosphorylated intermediates gradually decreased as a percentage of the total labeled products, coinciding with an increased labeling of the neutral (sucrose) and insoluble (starch) fractions (Fig. 3). However, after a 2-h chase, neutral and insoluble compounds accounted for more than 60% of the total $^{14}C$ incorporated by low photorespiration *Hydrilla*, as compared to less than 23% for the plants in the high photorespiratory state (Fig. 3). At all times during the chase, the high photorespiration plants incorporated a greater fraction of the label into the photorespiratory intermediates glycine and serine than did the low photorespiration plants. For example, after 2 h of chase, glycine and serine comprised 18% of the labeled products of the plants in the high photorespiratory state as compared with only 7% in the low photorespiratory state (Fig. 3).

For both the high and low photorespiration plants, total dpm incorporated during the pulse showed only a slight decrease during the chase period, even after a 2-h chase. This indicates that $^{14}C$ loss due to the excretion of labeled compounds such as glycolate must have been negligible, and that the per cent changes in the various compounds reflect actual dpm changes.

In contrast to *Hydrilla*, the carbon assimilation in low photorespiratory state *Myriophyllum* was almost exclusively via the Calvin cycle as $C_4$ acids were less than 10% of the initial labeled products while sugar-P and P-glycerate comprised 77% (Fig. 4). The fraction of $^{14}C$ incorporated into $C_4$ acids did not increase at low inorganic carbon concentrations, even when the equilibration and fixation were carried out at only 1 $\mu$M $HCO_3^-$ (data not shown). After a 20-s fixation period at 20 $\mu$M $H^{14}CO_3^-$, label in phosphorylated compounds decreased steadily during the chase period; this decrease coincided with a substantial increase in labeling of compounds in the insoluble and neutral fractions (Fig. 4).

No major differences in $C_4$ acid and sugar-P labeling patterns were evident when the low photorespiration *Myriophyllum* plants were treated with ethoxyzolamide to repress the photorespiratory

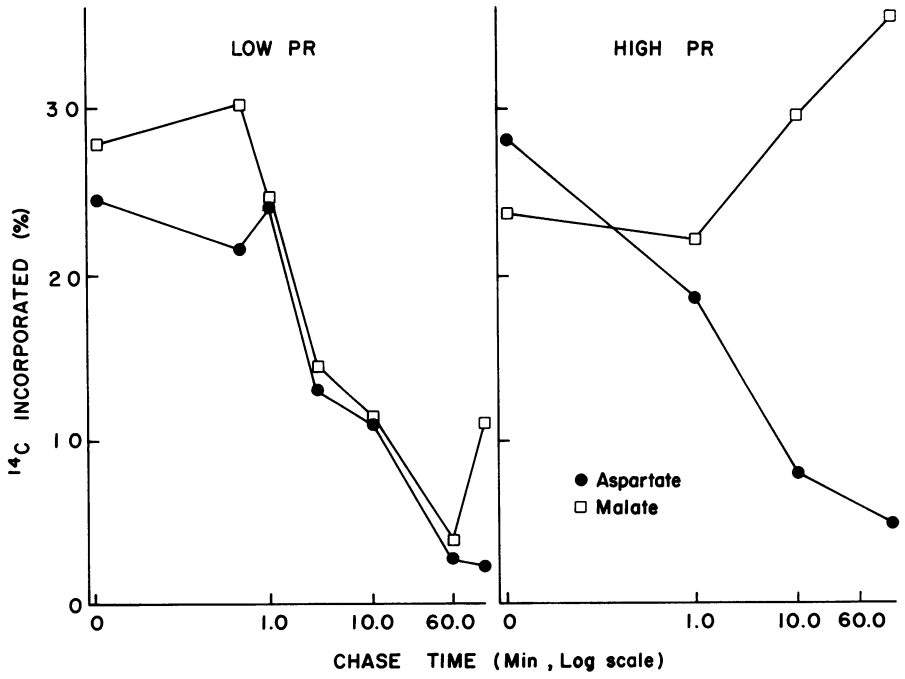


FIG. 1. Incorporation of $^{14}C$ into malate and aspartate for *Hydrilla* in the low and high PR states during a pulse-chase experiment. The zero time points represent the start of the $^{12}C$ chase following a 20-s $^{14}C$ pulse. Incorporation is expressed as a percentage of the total $^{14}C$ incorporated. The $^{14}C$ fixation rates were 12.8 ± 2.2 and 13.3 ± 1.5 $\mu$mol $CO_2$/mg Chl·h for low and high PR *Hydrilla*, respectively.

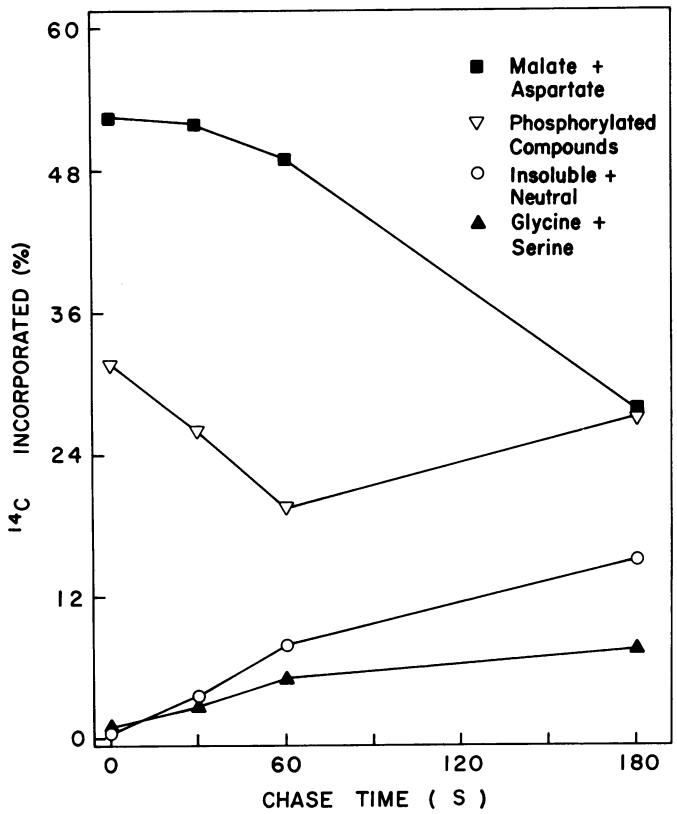


FIG. 2. Distribution of $^{14}C$ among the photosynthetic intermediates of *Hydrilla* in the low photorespiratory state during the initial period of the pulse-chase experiment. The zero time points represent the start of the $^{12}C$ chase following a 20-s $^{14}C$ pulse. Incorporation is expressed as a percentage of the total $^{14}C$ incorporated and the $^{14}C$ fixation rates were as in Figure 1.

state (Table I). However, *Myriophyllum* plants treated with ethoxyzolamide did exhibit an increased labeling of glycolate, glycine, and serine after a 20-s fixation period (Table I) and through 10 min of a subsequent chase (data not shown). Low photorespiration *Hydrilla* plants, in contrast, showed no affect of ethoxyzolamide on the short term labeling pattern of these photorespiratory intermediates or on the $C_4$ acids malate and aspartate (Table I). Sugar monophosphates did increase somewhat, apparently at the expense of P-glycerate and triose-P. For both *Hydrilla* and *Myriophyllum*, ethoxyzolamide treatment reduced the total $^{14}C$ incorporated by approximately 30%.

INH, an inhibitor of the glycine to serine conversion in photorespiration, can be used to block the flow of label through the photorespiratory pathway by inhibiting the further metabolism of glycine (24). When detached leaves of low photorespiration *Myriophyllum* were treated with increasing concentrations of INH, glycine represented an increasingly larger percentage of the $^{14}C$ incorporated (Fig. 5). At a saturating concentration of 10 mM INH, 30% of the $^{14}C$ incorporated by low photorespiration *Myriophyllum* was recovered in glycine as compared with less than 15% in the control (Fig. 5). As has been reported for terrestrial plants (24), total $^{14}C$ fixation decreased in plants treated with 10 mM INH to 70% of the control rate (Fig. 5). Unlike in terrestrial plants, however, serine did not decrease as a percentage of the $^{14}C$ incorporated. At 5 mM INH, the concentration used in subsequent experiments, excretion of organic $^{14}C$ by *Myriophyllum* was less than 3% of the total $^{14}C$ fixed, which was the same rate as the control (data not shown).

For *Myriophyllum* in both the high and the low photorespiratory state, 5 mM INH caused label to accumulate in glycine, apparently at the expense of neutral and acid 2 compounds (Table II). In the presence of INH, glycine represented a much greater proportion of the photosynthetically labeled products in high than in low photorespiration plants; the per cent values were 48 and 25, respectively (Table II). In the absence of INH, little difference in glycine labeling between the two photorespiratory states was evident (Table II). Similar, though less pronounced, effects of INH on glycine labeling were observed for *Hydrilla* plants in the low and high photorespiratory states (Table II).

When glycine accumulation was examined at 20 $\mu$M $HCO_3^-$ with respect to the $O_2$ concentration, at $O_2$ concentrations greater than 1 or 2% (gas phase), glycine comprised a greater fraction of the $^{14}C$-labeled products in high than in the low photorespiratory state of *Myriophyllum* (Fig. 6). At 80% $O_2$, glycine constituted only 25% of the total $^{14}C$ incorporated by the low photorespiration plants compared with greater than 45% in the high photorespiratory state (Fig. 6). In both photorespiratory states, formation of labeled glycine at 20 $\mu$M $HCO_3^-$ approached saturation at $O_2$ concentrations greater then 50%. When $HCO_3^-$ was held constant but the $O_2$ concentration was increased, glycine labeling increased with the increasing $[O_2]/[CO_2]$ ratio (Fig. 6, inset). In contrast, when measured under a constant $O_2$ concentration, glycine labeling in low photorespiration *Myriophyllum* was independent of the $HCO_3^-$ concentration, and therefore was not a true function of the $[O_2]/[CO_2]$ ratio (Fig. 6, inset).

A similar response pattern to that observed for glycine labeling was recorded for $O_2$ inhibition of net photosynthesis in low but not high photorespiration *Myriophyllum* plants. Thus, for plants in the low photorespiratory state, the per cent inhibition of net photosynthesis by 21% $O_2$ was independent of the $CO_2$ concentration and therefore inhibition of net photosynthesis by $O_2$ was not a true function of the external $[O_2]/[CO_2]$ ratio (Fig. 7). However, inhibition of net photosynthesis was affected by the $O_2$ concentration when measured under a constant $CO_2$ concentration (330 $\mu$l $CO_2$/L). In contrast to these results obtained with low photorespiration plants, the per cent inhibition of net photosynthesis by $O_2$ for *Myriophyllum* in the high photorespiratory state responded to changes in both $O_2$ and $CO_2$ and was thus a function of the external $[O_2]/[CO_2]$ ratio (Fig. 7). The per cent inhibition of net photosynthesis by $O_2$ was considerably less in the low photorespiration plants than in the high photorespiration plants whether measured under conditions of either constant $O_2$ or constant $CO_2$ (Fig. 7).

## DISCUSSION

Evidence for $C_4$ acid metabolism has been presented for a number of submersed angiosperm species based either on direct data from short and long term labeling studies (9, 11–13) or indirectly by implication from measurements of relatively high PEP carboxylase activities (7, 21). All of the angiosperms cited are monocotyledonous and five genera, *Egeria*, *Elodea*, *Hydrilla*, *Lagarosiphon*, and *Valisneria*, belong to one family, Hydrocharitaceae. Labeling studies with *Hydrilla* and *Egeria* have shown that the proportion of label incorporated into $C_4$ acids in the light increases as the level of inorganic carbon is decreased (8, 13). Thus, the formation of $C_4$ acids in photosynthesis by certain submersed angiosperm species is probably an adaptation to low external levels of inorganic carbon (30) and appears to be associated with the low photorespiratory state which is induced under these conditions (7, 13, 21), but which is inhibited when high $CO_2$ concentrations are used for growth (15).

In *Hydrilla*, although $C_4$ acids accounted for a similarly large percentage of the photosynthetically fixed carbon in both the low and the high photorespiratory states, differences in the fate of malate were apparent between the respective photorespiratory states. In low photorespiration plants, malate was utilized to

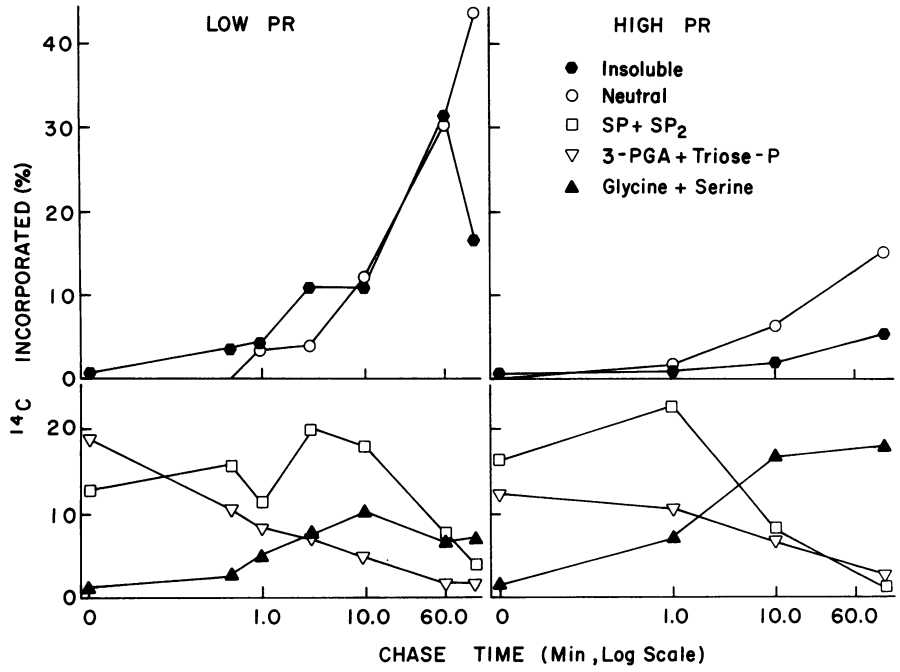


FIG. 3. Distribution of $^{14}C$ among the photosynthetic intermediates of *Hydrilla* in the low and high PR states during an extended pulse-chase experiment. The zero time points represent the start of the $^{12}C$ chase following a 20-s $^{14}C$ pulse. Incorporation is expressed as a percentage of the total $^{14}C$ incorporated and the $^{14}C$ fixation rates were as in Fig. 1. PGA, phosphoglyceric acid; SP, sugar-P.

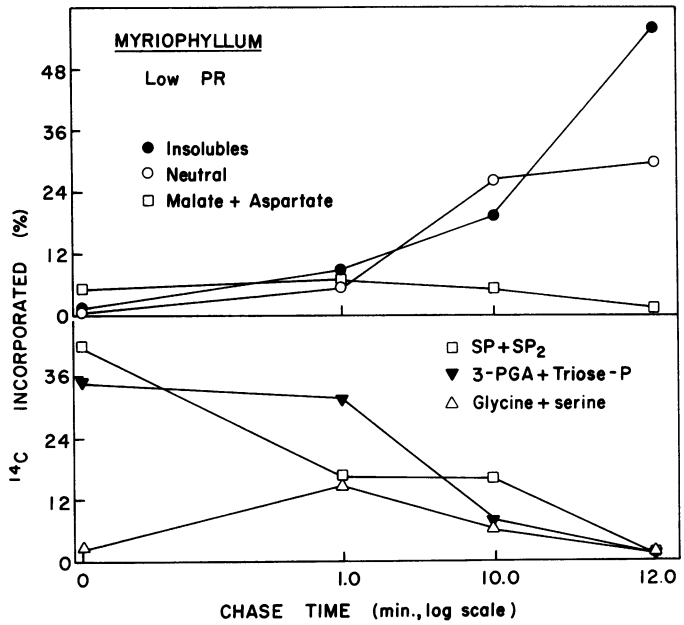


FIG. 4. Distribution of $^{14}C$ among the photosynthetic intermediates of *Myriophyllum* in the low PR state during an extended pulse-chase experiment. The zero time points represent the start of the $^{12}C$ chase following a 20-s $^{14}C$ pulse. Incorporation is expressed as a percentage of the total $^{14}C$ incorporated. The $^{14}C$ fixation rate was 11.0 ± 1.4 μmol $CO_2$/mg Chl·h. PGA, phosphoglyceric acid; SP, sugar-P.

form photosynthetic end products (sucrose and starch), probably as a result of the activities of $C_4$ pathway enzymes which increase with induction of the low photorespiratory state (21). This fixation and decarboxylation of $C_4$ acids in the light and the enzymic capacity to regenerate PEP via pyruvate Pi dikinase are features similar to the photosynthetic carbon metabolism in terrestrial $C_4$ plants (10), and in an analogous manner they may also function as a $CO_2$-concentrating mechanism in low photorespiration *Hydrilla*. The resulting increase in the concentration of $CO_2$ at the site of RuBP carboxylase-oxygenase could explain the $C_4$-like photosynthesis/photorespiration ratio which is characteristic of *Hydrilla* in the low photorespiratory state (7, 15, 21, 23). Unlike typical $C_4$ plants, P-glycerate and triose-P showed no initial increase in $^{14}C$ label as the chase commenced. This may be due to the lag period for loss of label from malate at the start of the chase (Fig. 1), which has been observed previously in pulse-chase studies with submersed plants (11, 13). Also, the possibility that some parallel (dual) carboxylation occurs cannot be excluded.

In contrast to the data for the low photorespiration state, in the high photorespiratory state of *Hydrilla*, label accumulated in malate throughout an extended chase period and did not show any evidence of turnover. This lack of malate turnover during photosynthesis is probably the result of the low activities of $C_4$ enzymes in the high photorespiration plants (21), It is nonetheless unusual, considering the large percentage of carbon that was incorporated into this compound. Similar results, however, have been reported for *Egeria* plants with high $CO_2$ compensation points (9). In these *Egeria* plants, the malate pool expands during the light period and decreases in the dark, which suggests that malate formed in the high photorespiratory state is an end product, and is used for some function such as ion balance (9, 12), even possibly to facilitate $HCO_3^-$ uptake, rather than as a source of $CO_2$ for RuBP carboxylase-oxygenase.

In the low photorespiratory state, the available evidence with *Hydrilla* indicates that the malate pool fluctuates with an opposite diel rhythm, as higher levels of titratable acidity are measured after a dark than after a light period (13). It is likely that the titratable acidity of low photorespiration *Hydrilla* plants increases during the dark period due to their relatively high capacity for dark $CO_2$ fixation (13, 15), and even though these plants have the potential for malate production during the light period, the low $CO_2$ availability in a natural daytime environment could limit malate formation and result in a net reduction in this compound during the day. This scheme for malate metabolism

Table I. *Effect of Ethoxyzolamide (100 μM) on the Distribution of $^{14}C$ among the Photosynthetic Intermediates of Myriophyllum and Hydrilla in the Low Photorespiratory State*

Plants were pulsed for 20 s at 20 μM $H^{14}CO_3^-$ and 21% $O_2$ (gas phase). The rates of $^{14}C$ fixation for control and ethoxyzolamide-treated plants were 13.8 and 10.0 μmol $CO_2$/mg Chl·h, respectively, for *Myriophyllum*, and 13.6 and 9.1 μmol $CO_2$/mg Chl·h, respectively, for *Hydrilla*.

| Fraction | *Myriophyllum* | | *Hydrilla* | |
|---|---|---|---|---|
| | Control | Ethoxyzolamide | Control | Ethoxyzolamide |
| | % $^{14}C$ incorporated | | | |
| Basic | 6.7 | 9.2 | 30.3 | 23.8 |
| Aspartate | 1.4 | 0.6 | 24.6 | 18.8 |
| Alanine | 0.4 | 0.6 | 1.0 | 0.7 |
| Glycine + serine | 2.8 | 7.1 | 1.0 | 1.1 |
| Acid 1 | 7.0 | 9.8 | 33.5 | 41.1 |
| Malate | 4.0 | 4.5 | 27.9 | 31.8 |
| Glycolate | 1.1 | 4.8 | 0.4 | 0.4 |
| Acid 2 | 84.1 | 81.0 | 35.4 | 32.2 |
| Sugar-P | 35.8 | 23.2 | 6.3 | 14.5 |
| Sugar-$P_2$ | 19.1 | 24.8 | 6.6 | 7.3 |
| P-glycerate + triose-P | 27.3 | 25.2 | 18.9 | 5.8 |
| Insoluble | 1.8 | 1.8 | 0.8 | 2.8 |

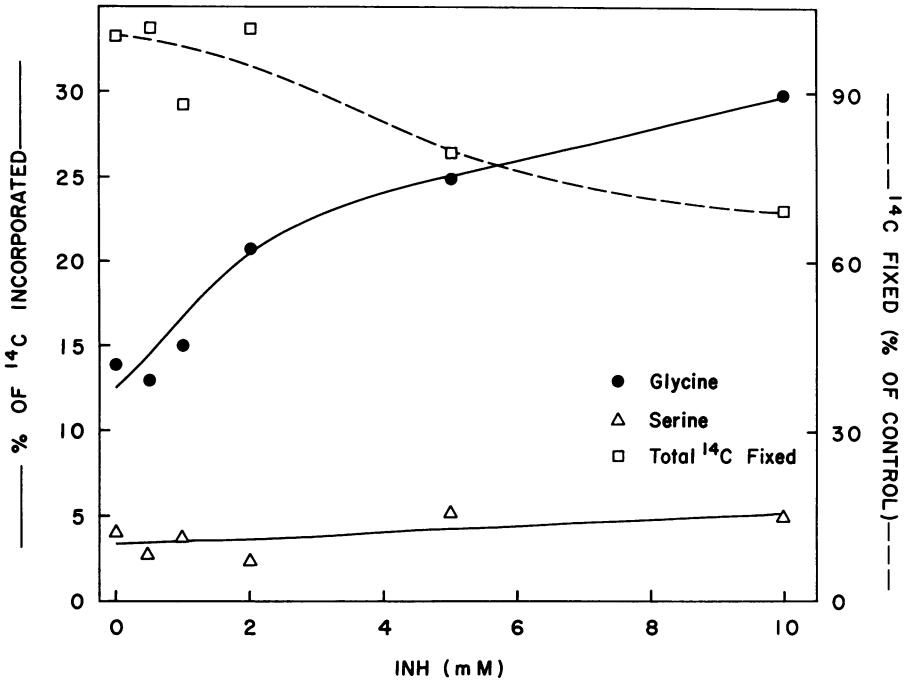


FIG. 5. The effect of INH concentration on total $^{14}C$ fixation and the incorporation of $^{14}C$ into glycine and serine for *Myriophyllum* in the low PR state at 20 μM $H^{14}CO_3^-$ and 50% $O_2$ (gas phase). Incorporation into glycine and serine at each INH concentration is expressed as a percentage of the total $^{14}C$ fixed in 15 min at that INH concentration. The 100% (control) $^{14}C$ fixation rate without INH was 11.3 μmol $CO_2$/mg Chl·h.

in low, but not in high, photorespiration plants is somewhat reminiscent of terrestrial CAM plants, and diurnal acid patterns similar to that reported by Holaday and Bowes (13) for *Hydrilla* have since been reported for the submersed lower vascular plant *Isoetes* (17) and the submersed angiosperm *Scirpus* (5).

To date, at least three genera of aquatic angiosperms (*Myriophyllum*, *Ceratophyllum*, and *Ranunculus*) are known to lack significant $C_4$ acid metabolism based either on $C_3$-type labeling patterns (27) or on low levels of PEP carboxylase activity (21, 29, 30). The pulse-chase labeling data presented here confirm the $C_3$-type labeling patterns for *Myriophyllum*. Two of these three genera, *Myriophyllum* and *Ceratophyllum*, are known to exhibit variable photorespiration states (15, 21), like *Hydrilla*, despite the lack of a $C_4$ acid-type mechanism to achieve the low photorespiratory state. In this regard, they seem to be similar photosynthetically to low $CO_2$-grown unicellular green algae (3, 4). As with the algae, the mechanism involved in reducing photorespiration in these submersed angiosperms appears to depend, in part, on the activity of carbonic anhydrase as demonstrated by the manner in which the carbonic anhydrase inhibitor ethoxyzolamide altered the gas exchange characteristics (23), and increased the labeling of photorespiratory intermediates in low photorespiration *Myriophyllum* plants (Table I).

Low $CO_2$-grown algae probably utilize an inorganic carbon-accumulating system in conjunction with the carbonic anhydrase activity, in order to concentrate $CO_2$ at the active site of fixation by RuBP carboxylase-oxygenase (3, 4, 26). The labeling data in the present study are consistent with the operation of a similar system in *Myriophyllum* in the low photorespiratory state. First, the increase in glycine labeling which occurred in the presence

Table II. *The Effect of Isonicotinic Acid Hydrazide (5 mM) on the Distribution of $^{14}C$ among the Photosynthetic Intermediates of Myriophyllum and Hydrilla in the Low and High Photorespiratory States*

Plants were exposed to $^{14}C$ in the light for 15 min at 20 μM $H^{14}CO_3^-$ and 50% $O_2$ (gas phase). The rates of $^{14}C$ fixation for control and INH-treated *Myriophyllum* were 11.3 and 9.0 μmol $CO_2$/mg Chl·h, respectively, for low photorespiration plants, and 5.4 and 3.3, respectively, for high photorespiration plants. For control and INH-treated *Hydrilla*, the rates were 4.2 and 2.9 μmol $CO_2$/mg Chl·h, respectively, for low photorespiration plants, and 3.5 and 2.9, respectively, for high photorespiration plants.

| Fraction | *Myriophyllum* | | | | *Hydrilla* | | | |
|---|---|---|---|---|---|---|---|---|
| | Low PR | | High PR | | Low PR | | High PR | |
| | Control | INH | Control | INH | Control | INH | Control | INH |
| | % $^{14}C$ incorporated | | | | | | | |
| Basic | 34.4 | 48.7 | 23.8 | 77.2 | 23.2 | 33.2 | 33.2 | 58.7 |
| Aspartate | 4.8 | 5.4 | 0.7 | 7.6 | 5.8 | 10.0 | 11.6 | 26.1 |
| Glycine | 14.0 | 25.4 | 11.0 | 47.5 | 5.8 | 9.5 | 6.1 | 13.7 |
| Serine | 4.2 | 7.9 | 3.6 | 10.0 | 1.6 | 1.8 | 4.6 | 2.3 |
| Neutral | 25.6 | 19.5 | 11.8 | 5.7 | 21.2 | 11.2 | 5.8 | 4.0 |
| Acid 1 | 8.8 | 5.0 | 13.2 | 2.3 | 25.5 | 25.8 | 42.5 | 26.1 |
| Acid 2 | 29.3 | 16.9 | 49.1 | 12.5 | 24.4 | 20.1 | 16.6 | 10.4 |
| Insoluble | 1.9 | 9.7 | 2.6 | 2.3 | 4.4 | 7.6 | 1.7 | 1.4 |

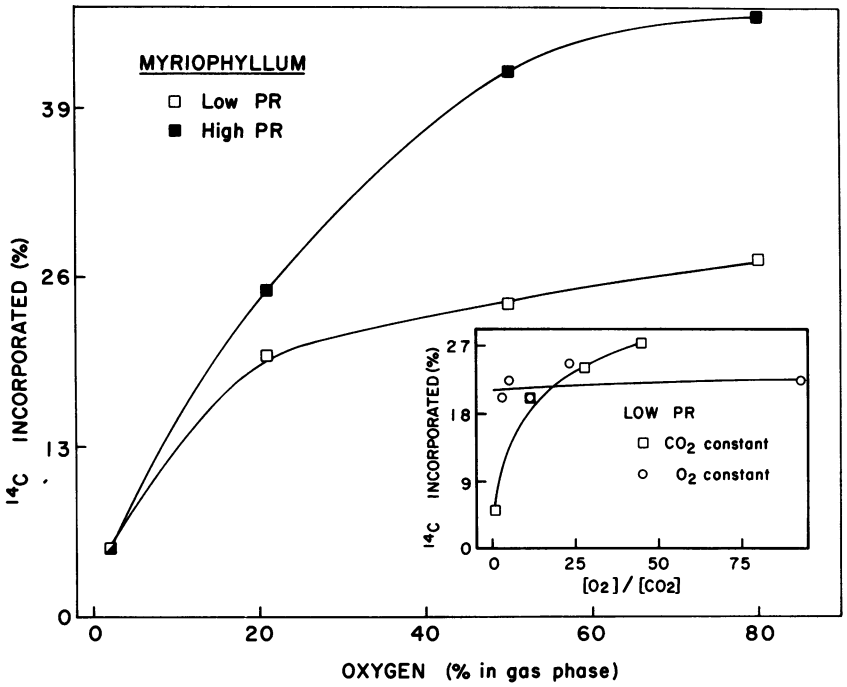


FIG. 6. The effect of $O_2$ concentration on the incorporation of $^{14}C$ into glycine for *Myriophyllum* in the low and high PR state after 15 min at 20 μM $H^{14}CO_3^-$ and 5 mM INH. Incorporation is expressed as a percentage of the total $^{14}C$ incorporated at each $O_2$ concentration. Inset: the effect of the external $O_2/CO_2$ concentration ratio on the incorporation of 14C into glycine for *Myriophyllum* plants in the low PR state after photosynthesis in 5 mM INH. Experiments were performed either at a constant $H^{14}CO_3^-$ concentration of 20 μM with $O_2$ varying from 1 to 80% (gas phase), or at a constant $O_2$ concentration of 21% (gas phase) with varying $H^{14}CO_3^-$ concentrations ranging from 2.5 to 100 μM.

of INH suggests that the low photorespiratory state of *Myriophyllum* is not the result of an alternate pathway that bypasses the reactions involved in the release of photorespiratory $CO_2$. Second, the reduced label in glycine of low, as compared to high, photorespiration plants in the presence of INH suggests that less carbon passes through the photorespiratory pathway, as opposed to there being an increase in refixation of photorespired $CO_2$ in the low photorespiration plants. Both of these lines of evidence support the contention that the reduction of photorespiratory $CO_2$ release in low photorespiration *Myriophyllum* is due to elevated $CO_2$ at the site of RuBP carboxylase-oxygenase. Similar arguments can be made for low photorespiration *Hydrilla* from the inhibitor and labeling data presented in this study.

When evaluated as a function of changing $O_2$ but constant inorganic carbon concentrations, at any given $[O_2]/[CO_2]$ ratio, the percentage of $^{14}C$ incorporated into glycine in high photorespiration *Myriophyllum* was virtually identical to the percentage reported for leaf cells isolated from the $C_3$ plant, soybean, treated similarly with INH (24). In contrast, glycine labeling in low photorespiration *Myriophyllum* was considerably less. As reported for the soybean cells (24), increasing $O_2$ increased the label entering glycine, irrespective of the photorespiratory state of the *Myriophyllum*.

The results of the experiments, in which the $O_2$ was held constant and the inorganic carbon level was varied, indicate that at a given $O_2$ concentration the per cent of glycine labeling and the per cent of $O_2$ inhibition of net photosynthesis in low photorespiration *Myriophyllum*, by being responsive only to

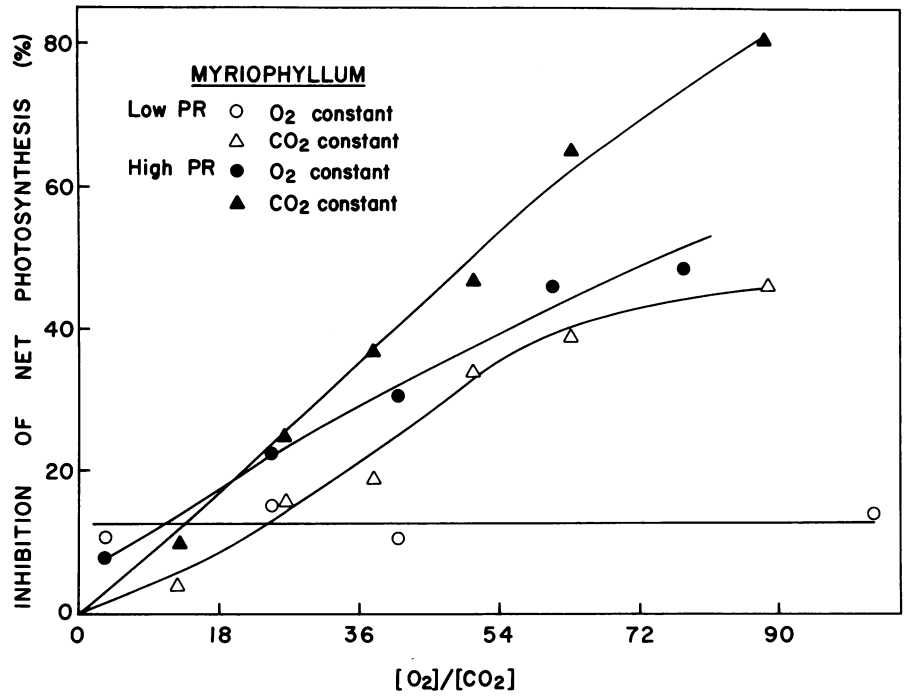


FIG. 7. The effect of the external $O_2/CO_2$ concentration ratio on the per cent inhibition of net photosynthesis by $O_2$ for *Myriophyllum* plants in the low and high PR state. Inhibition was measured as a decrease from the net photosynthetic rate at 1% $O_2$ (gas phase). IR gas analysis measurements were made either at 327 $\mu$l $CO_2$/L and from 1 to 80% $O_2$ (constant $CO_2$), or 21% $O_2$ and from 50 to 2500 $\mu$l $CO_2$/L (constant $O_2$) gas concentrations.

changes in the $O_2$ concentration, are not strictly a function of the $[O_2]/[CO_2]$ ratio. Shelp and Canvin (25) have reported a similar response in low $CO_2$-grown *Chlorella* cells. These authors suggested that the $O_2$ inhibition of net photosynthesis exhibited by *Chlorella* may not be photorespiratory in nature. However, this cannot be the case for *Myriophyllum*, because when the $O_2$ concentration was raised, the increased $O_2$ inhibition of net photosynthesis was accompanied by an increase in the labeling of the photorespiratory intermediate glycine.

These gas exchange and labeling characteristics exhibited by *Myriophyllum* plants in the low photorespiratory state, in response to changes in the external $CO_2$ and $O_2$, superficially appear to be inconsistent with the properties of RuBP carboxylase-oxygenase. *In vitro* determinations of the $v_o/v_c$ ratio of the enzyme from low photorespiration *Myriophyllum* using several $CO_2$ and $O_2$ concentrations indicated that the relative activity of the carboxylase and oxygenase is a linear function of the $[O_2]/[CO_2]$ ratio as is the case for RuBP carboxylase-oxygenase from other sources (16), including high photorespiration *Myriophyllum* (M. E. Salvucci and G. Bowes, unpublished data). Thus, for *Myriophyllum* in the low photorespiratory state, the observed physiological responses of photosynthesis and photorespiration, which are catalyzed by RuBP carboxylase and oxygenase, respectively, are probably not due to any kinetic changes in the two activities of this enzyme, but rather appear due to an uncoupling of the enzyme from the external $[O_2]/[CO_2]$ ratio. Because the diffusion of $CO_2$ and $O_2$ into the active site of the RuBP carboxylase-oxygenase is one of the factors determining the rates of photosynthesis and photorespiration, it may be possible that carbonic anhydrase, in association with a $HCO_3^-$ accumulation system, modulates the internal pool of inorganic carbon (which exists in both the $HCO_3^-$ and free $CO_2$ form) so as to act as a "buffer" for free $CO_2$. This could to some extent uncouple $CO_2$ at the site of carboxylation from the external $CO_2$ concentration, without affecting the access of external $O_2$ to the oxygenation site. The observed physiological responses could then still be attributed to RuBP carboxylase-oxygenase but in association with the accumulation/utilization mechanism dependent upon carbonic anhydrase. Further evidence for this hypothesis comes from the observation that both *Myriophyllum* and *Hydrilla* appear to be capable of utilizing $HCO_3^-$ ions in the bathing medium, even though free $CO_2$ is the preferred form (30).

Under certain conditions, carbonic anhydrase has been shown to enhance $CO_2$ fixation by isolated RuBP carboxylase and this enhancement (as a per cent) increases with decreasing $CO_2$ concentration (6 ). Tsuzuki *et al.* (28) have observed a similar enhancement effect on photosynthetic $CO_2$ fixation by adding carbonic anhydrase exogenously to *Chlorella* cells. These investigators have postulated that, internally, carbonic anhydrase facilitates the utilization of an elevated $HCO_3^-$ pool in the chloroplasts as an indirect source of $CO_2$ which can enhance photosynthesis to the detriment of photorespiration (28). Carbonic anhydrase may function in a somewhat similar way in the photorespiratory state of submersed angiosperms as evidenced by the enhanced ethoxyzolamide inhibition of net photosynthesis at low $CO_2$ concentrations for *Myriophyllum* and *Hydrilla* in the low, but not the high, photorespiratory state (23).

Thus, it appears likely that both $C_4$ acid- and non-$C_4$ acid-dependent systems for concentrating $CO_2$ occur among submersed aquatic angiosperms, which in conjunction with carbonic anhydrase, reduce the activity of the photorespiratory carbon oxidation cycle and enhance the conservation of carbon in environments with low daytime inorganic carbon levels (30). As with other autotrophic organisms, both systems eventually depend on the operation of the $C_3$ photosynthetic carbon reduction cycle for the formation of carbohydrates. However, for $C_4$ plants, unicellular green algae, or cyanobacteria, possession of the $C_3$ photosynthetic carbon reduction cycle is not in itself a sufficient basis to categorize these organisms as $C_3$ plants. A similar argument can be made for submersed aquatic macrophytes, as their variable photorespiratory capacity, and the associated biochemical modifications, support the contention that they cannot be categorized within any of the photosynthetic groups currently recognized.

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