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thesis entitled

Carbonic Anhydrase Levels and Internal Lacunar

CO_2 Concentrations in Aquatic Macrophytes

presented by

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has been accepted towards fulfillment
of the requirements for

M.Sc. degree in Botany

Major professor
(Robert G. Wetzel)

Date 13 February 1979

CARBONIC ANHYDRASE LEVELS AND INTERNAL LACUNAR CO_2 CONCENTRATIONS IN AQUATIC MACROPHYTES

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1979

ABSTRACT

CARBONIC ANHYDRASE LEVELS AND INTERNAL LACUNAR CO_2 CONCENTRATIONS IN AQUATIC MACROPHYTES

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Carbonic anhydrase levels were examined in a variety of aquatic macrophytes from different habitats. In general, carbonic anhydrase levels increased across the habitat gradient such that activities were low in submersed aquatic macrophytes and high in emergent macrophytes with floating-leaved and free-floating plants exhibiting intermediate activities. Internal lacunar CO_2 concentrations were analyzed in relation to carbonic anhydrase activities. There was no correlation between these two parameters. Internal CO_2 concentrations ranged from low to high in submersed macrophytes, but were low in floating-leaved and emergent macrophytes. The observed internal CO_2 concentrations are discussed in relation to the individual morphologies of the plants and the environments in which they occurred.

ACKNOWLEDGMENTS

I extend appreciation and thanks to my committee members, Drs. R. G. Wetzel, C. J. Pollard, and P. A. Werner for critical evaluation of the manuscript. In particular, I wish to thank Dr. R. G. Wetzel for his assistance in the development and completion of this project and Dr. C. J. Pollard for his helpful suggestions regarding methods.

Many people aided me tremendously in my everyday research problems and in providing technical expertise and assistance. I wish to thank Wilson Cunningham, Joyce Dickerman, Jim Grace, Gordon Godshalk, Polly Penhale, Art Stewart, and Amy Ward for helpful discussions and Pat Brown and Joy Sonnad for technical assistance. I also sincerely thank Anita Johnson and Marian Weaver for help in preparation of the manuscript.

I particularly want to thank Claude Leblanc for his continual encouragement and understanding throughout the course of this project.

Financial support for this study was provided by the Department of Energy EY-76-S-1599 (C00-1599-147).

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INTRODUCTION

Carbon dioxide plays an instrumental role in the life of all organisms. During photosynthesis, CO_2 is assimilated by plants and reduced to carbohydrates. Oxidation of these carbohydrates occurs in all organisms during respiration and CO_2 is once more released. Carbon dioxide also is involved in buffering pH changes in some tissues through the CO_2-HCO_3^- buffering system. In this system (Wetzel, 1975), as atmospheric CO_2 dissolves in water, it slowly hydrates to carbonic acid. Carbonic acid then immediately dissociates into bicarbonate and a proton.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Below pH 5, free CO_2 dissolved in water dominates and between pH 7 and 9, the equilibrium tends toward bicarbonate. At a pH > 9.5, the dissociation of bicarbonate to carbonate becomes significant. This latter reaction, however, can be virtually ignored in biological systems where the pH is usually less than 8 (Edsall and Wyman, 1958).

The hydration of CO_2, as well as the dehydration of carbonic acid, occur relatively slowly--too slow to be

implemented effectively in a buffering capacity and too slow to supply adequate levels of CO_2 to biochemical reactions (Edsall and Wyman, 1958). The enzyme carbonic anhydrase (E.C.4.2.1.1. carbonate hydrolyase) greatly accelerates these processes and may potentially double the reaction rate (Davis, 1963; Edsall and Wyman, 1958; Lindskog et al., 1971; Waygood, 1955). The turnover number for carbonic anhydrase isolated from spinach leaves is 40-80 mM CO_2 hydrated min^{-1} $\mu mole^{-1}$ carbonic anhydrase (Jacobson et al., 1975). In addition to the ability to catalyze the reversible hydration of CO_2, animal carbonic anhydrase is able to catalyze the hydrolysis of esters (Malmström et al., 1964; Pocker and Meany, 1967) and the hydration of aldehydes (Pocker and Meany, 1967). Plant carbonic anhydrase, however, is unable to hydrolyze esters (Tobin, 1970) and can only weakly hydrate aldehydes (Kisiel and Graf, 1972; Tobin, 1970). Jacobson et al. (1975) also suggest that carbonic anhydrase binds 3-phosphoglyceric acid, implying a regulatory role in the pentose phosphate reductive pathway of photosynthetic metabolism. But, the significance of this is probably small in view of the enzyme's weak ability to hydrate aldehydes.

Carbonic anhydrase is present in virtually all organisms: in plants, animals, and bacteria (Lindskog et al., 1971). In the plant kingdom, carbonic anhydrase is present in both freshwater and marine algae (Bowes, 1969; Graham and Smillie, 1976; Ikemori and Nishida, 1968; Ingle and Colman,

1975; Lichtfield and Hood, 1964), in bryophytes (Brown and Eyster, 1955; Steemann Nielsen and Kristiansen, 1949), in pteridophytes and gymnosperms (Graham et al., 1974), and in monocotyledonous and dicotyledonous vascular plants (Atkins et al., 1972a, b; Chen et al., 1970; Everson and Slack, 1968). The enzyme is present only in leaves and is not present in roots, although the enzyme is present in the root nodules of leguminous species (Atkins, 1974). In this last case, carbonic anhydrase apparently is synthesized in the plant root when the bacterium Rhizobium infects the root and induces formation of a nodule. Virtually all of the enzymatic activity (99%) found to be present is in the root nodule. No activity is associated with intact bacteroids isolated from the nodule, although a very slight activity is detected in disrupted bacteroids. The author hypothesizes that the enzyme functions to aid transport of respired CO_2 out of the nodules.

Plant carbonic anhydrase is a zinc-containing metalloprotein. The zinc atom appears to be tightly bound to the apoenzyme and its presence is required for enzymatic activity (Tobin, 1970). Werber (1976) postulates that the zinc atom acts as a carrier for hydroxyl ions in the catalysis of CO_2 hydration as follows:

$$E\text{-}Zn^{++}\text{-}OH^{-} + CO_2 \rightleftarrows E\text{-}Zn^{++}\text{-}OH^{-}\text{-}CO_2 \rightleftarrows E\text{-}Zn^{++}\text{-}HCO_3^{-}$$

$$E\text{-}Zn^{++}\text{-}HCO_3^{-} + H_2O \rightleftarrows E\text{-}Zn^{++}\text{-}H_2O + HCO_3^{-}$$

The metal-bound water molecule would be catalytically ionized after each turnover to regenerate the OH^- carrying active center of the enzyme:

$$E\text{-}Zn^{++}\text{-}H_2O + B \rightleftarrows E\text{-}Zn^{++}\text{-}OH^- + BH^+$$

where B is a buffer acceptor. The CO_2 dehydration reaction would occur in the reverse order.

Plant carbonic anhydrase contains sulfhydryl groups, which in most cases must be stabilized in tissue homogenates by the addition of reducing agents. Bradfield (1947) originally discovered that the addition of cysteine to his buffer system increased the observed carbonic anhydrase activity of plant extracts. Upon standing in the absence of cysteine the enzyme rapidly lost activity. This observation was supported by later evidence that p-chloromercuribenzoate (PCMB), iodobenzoate, and azide, all fairly specific inactivators of sulfhydryl groups, inhibit enzymatic activity in a number of different kinds of plants (Bradfield, 1947; Everson, 1970; Kiesel and Graf, 1972; Sibly and Wood, 1951). In the presence of PCMB or sodium arsenite, the enzyme was reactivated with the addition of reduced glutathione or cysteine (Sibly and Wood, 1951). There appears to be some variation, however, in the effect of sulfhydryl group inhibitors on carbonic anhydrase activity. Everson (1971) showed that PCMB and arsenite at 10^{-3} M inhibit enzymatic activity almost completely in two C_4 plant species (Zea mays and Amaranthus viridis), but inhibit carbonic anhydrase hardly at

all in Spinacea oleracea (spinach), a C_3 species. In agreement with these data, Pocker and Ng (1973) reported that a reducing agent was not needed for stabilization of the enzyme in spinach. The use of a phosphate-NaCl-EDTA buffer resulted in very little loss of enzymatic activity after 50 hours at room temperature whereas the use of a phosphate buffer containing the reducing agent, 2-mercaptoethanol, resulted in a rapid decrease in enzymatic activity over the same period of time. Use of 5,5'-dithiobis(2-nitrobenzoate) (Nbs_2) in these experiments delineated more precisely the nature of the sulfhydryl groups of the enzyme. Nbs_2 is a reagent which specifically oxidizes sulfhydryl groups. When Nbs_2 was added to an enzyme solution in the absence of a reducing agent, no loss of enzymatic activity and no reduction of the Nbs_2 occurred. Reduction of Nbs_2 did occur, however, when the enzyme complex was dissociated with 6 M guanidine hydrochloride. Pocker and Ng's interpretation of these results was that in the intact enzyme, or undissociated form, the sulfhydryl groups were located internally in the enzyme complex and functioned to maintain the structural integrity of the enzyme. When the enzyme complex was dissociated with the addition of guanidine hydrochloride, the sulfhydryl groups became exposed and were reduced by the Nbs_2. They noted that PCMB also can denature proteins and hence could have inactivated carbonic anhydrase in previous studies by causing the molecule to partially dissociate. This dissociation would have revealed sulfhydryl groups and in the

absence of a reducing agent left them open for possible oxidation by other substances in the plant extracts. The actual presence of sulfhydryl groups in plant carbonic anhydrase is supported by amino acid analyses by Kiesel and Graf (1972) and Tobin (1970). Both found the sulfhydryl-containing amino acid cysteine to be a constituent of the enzyme.

The structure of the enzyme differs between monocotyledonous and dicotyledonous vascular plants and this difference may partially account for the variability observed above. Carbonic anhydrase isolated from the monocotyledon Tradescantia albiflora has an approximate molecular weight of 42,000, a subunit size of 27,500, and contains one mole of zinc per 34,000 g of protein (Atkins et al., 1972b). In contrast, carbonic anhydrase from dicotyledonous species appears to be a hexameric enzyme with a molecular weight of about 180,000 (range of 180,000 to 205,000), a subunit size of about 30,000, and contains six zinc atoms per molecule (Atkins, 1974; Atkins et al., 1972b; Kiesel and Graf, 1972; Pocker and Ng, 1973; Tobin, 1970). The enzyme isolated from leguminous root nodules has a molecular weight of 45,000 and perhaps originates as a subunit of the dicotyledonous enzyme (Atkins, 1974). These differences in size of the enzyme between monocotyledons and dicotyledons may partially account for the difference in stability of the enzyme to sulfhydryl group inactivators. The larger size of the dicotyledonous enzyme may mean that the sulfhydryl groups of the

subunits are bound up to a greater degree in sulfhydryl group interactions than is true for the smaller monocotyledonous enzyme. Hence, the observation by Everson (1971) that Zea mays is much more sensitive to sulfhydryl group inactivators than is spinach. This explanation, however, would not suffice for Everson's observation of the sensitivity of Amaranthus viridus, a dicotyledon, to sulfhydryl group inhibitors.

Further variability in the structure of carbonic anhydrase is expressed within monocotyledonous and dicotyledonous plants and algae as Atkins et al. (1972b) and Graham et al. (1971) demonstrated the existence of carbonic anhydrase isoenzymes using polyacrylamide gel electrophoresis. Kachru and Anderson (1974) were able to isolate chloroplastic and cytoplasmic enzymes from Pisum sativum L. by use of isoelectric focusing. The existence of carbonic anhydrase isoenzymes led to the question of the intracellular localization of carbonic anhydrase. The majority of enzymatic activity has been shown to reside in the stroma of the chloroplast. Poincelot (1972a) determined that 63 percent of the carbonic anhydrase activity in spinach is in the chloroplast. The remaining portion was shown to exist outside of the chloroplast, most likely in the cytoplasm. By isolation of intact chloroplast envelope and lamellar membranes, Poincelot also demonstrated that 95.4 percent of the total enzymatic activity was located in the stromal fraction of the chloroplast as opposed to the lamellar membrane

fraction. In addition, he found a strong correlation between carbonic anhydrase activity and ribulose bisphosphate carboxylase (RuBPcase) activity, again indicating that the enzyme was located in the stroma. Jacobson et al. (1975) confirmed these results in their studies of spinach. They showed a positive correlation between the distribution of carbonic anhydrase and glyceraldehyde 3-phosphate dehydrogenase, a chloroplastic marker enzyme. No carbonic anhydrase activity was found in either mitochondria or microbodies. They concluded that most, if not all, of the enzyme was located in the chloroplast. Jacobson et al. also found carbonic anhydrase to be localized in the stroma of the chloroplast.

The above studies used spinach, a C_3 plant, to determine the intracellular location of the enzyme and did not consider C_4 plants. Everson and Slack (1968) carried out a comparative study of C_3 and C_4 plants in which the levels of carbonic anhydrase and the intracellular localization of the enzyme in plants from these two groups were examined using a non-aqueous extraction technique to prevent the loss of enzymes from isolated chloroplasts. Everson and Slack correlated carbonic anhydrase activity with either RuBPcase as a marker for chloroplastic constituents or with acid phosphatase as a marker for cytoplasmic elements. In two C_3 plant species, Spinacia oleracea and Pisum sativum, the bulk of carbonic anhydrase activity was associated with RuBPcase activity and with the pattern of chlorophyll

distribution, indicating that the carbonic anhydrase activity was chloroplastic in origin. Only a small percentage of carbonic anhydrase activity was found in the same fractions as acid phosphatase. The distribution of carbonic anhydrase in the two C_4 species, Zea mays and Amaranthus palmeri, was less clear-cut even though Everson and Slack concluded that carbonic anhydrase was located in the cytoplasm of C_4 plants. Although slightly more carbonic anhydrase activity was detected in the cytoplasm, some activity was also found in the chloroplast. In addition, the carbonic anhydrase activity of the C_4 plants was only 10% - 20% of the activity observed in C_3 plants. Graham et al. (1971) demonstrated further that the bulk of carbonic anhydrase activity in C_4 plants was in the mesophyll cells and that very little activity was associated with the bundle sheath cells.

Poincelot (1972b), however, presented evidence that the levels of carbonic anhydrase in Zea mays (maize) is comparable to those in spinach by use of a progressive grinding technique. This technique purportedly gives more complete extraction of enzymes from leaves which are difficult to completely homogenize, such as maize leaves. Poincelot also found that 85% - 90% of the carbonic anhydrase activity in maize is associated with the mesophyll cells. He attributed the low level of carbonic anhydrase activity that was observed in the bundle sheath cells to be a result of contamination from mesophyll cells. In contrast to Graham et al. (1971), Poincelot determined that the carbonic

anhydrase activity of the mesophyll cells was confined to the chloroplast rather than to the cytoplasm. Poincelot's study, however, did not present sufficient data to decisively conclude that carbonic anhydrase was located in the chloroplasts of mesophyll cells. In summary, C_3 and C_4 plants have comparable levels of carbonic anhydrase. The carbonic anhydrase activity is contained within the stroma of the chloroplast in C_3 plants. In C_4 plants, carbonic anhydrase activity is restricted to the mesophyll cells, but the exact intracellular location has yet to be determined.

The functional role of carbonic anhydrase in plants has not been definitely established. Most evidence points to a relationship between carbonic anhydrase activity and photosynthetic capacity. Several theories have developed which attempt to delineate the function of carbonic anhydrase in plants and several methodological approaches have been utilized. Each of these methods, however, has its drawbacks; interpretation of results may be difficult because of the simultaneous presence of complicating variables or because of technical problems.

Burr (1936) was the first to propose that carbonic anhydrase functions in photosynthesis. One of the first physiological experiments that related carbonic anhydrase activity to photosynthesis was done by Nelson et al. (1969). They observed that Chlamydomonas reinhardtii grown on atmospheric levels of CO_2 (0.03%) contained 10-20 times greater carbonic anhydrase activity than cells grown on air

supplemented with 1% CO_2. Similar results were observed by Graham et al. (1971) for Chlorella pyrenoidosa and Chlamydomonas reinhardi, and by Ingle and Colman (1975) for four species of blue-green algae when the algae were grown on air and on air plus 5% CO_2. In addition, Graham et al. (1971) found that when Chlorella pyrenoidosa was grown on air plus 5% CO_2 (low carbonic anhydrase) and then transferred to air, the alga was unable to photosynthesize until the levels of carbonic anhydrase rose. In this experiment, the carbonic anhydrase levels increased 100-fold after an induction period of 90 minutes and was accompanied by an 8-fold increase in photosynthetic oxygen evolution. During the induction period, neither the enzymes of the reductive pentose phosphate pathway nor of β-carboxylation changed. Reed and Graham (1977) also found carbonic anhydrase levels to be higher in air-grown Chlorella pyrenoidosa than in cells grown in 5% CO_2. They noted that levels of other enzymes of the reductive pentose phosphate pathway remained the same at both CO_2 concentrations.

Other evidence, however, points out that algal cells grown under different CO_2 conditions actually differ physiologically in ways other than in levels of carbonic anhydrase activity. CO_2 content during growth had no effect on RuBPcase activity, but did affect levels of phosphoenolpyruvate carboxylase (PEPcase), malic enzyme, catalase, malate dehydrogenase, glycolate dehydrogenase, serine-pyruvate aminotransferase, and aspartate-α-ketoglutarate

aminotransferase in Anacystis nidulans (strain L 1402-1) (Döhler, 1974). Except for RuBPcase and PEPcase, none of these enzymes were investigated by Reed and Graham (1977). Lonergan and Sargent (1978) observed differences between Euglena gracilis grown on air and those grown on 5% CO_2 in whole cell chlorophyll a fluorescence transients and in 2,6-dichlorophenolindophenol (DCPIP) reduction. The change in fluorescence transients indicated that a change in photosystem orientation had occurred. DCPIP reduction was about four times higher in chloroplasts isolated from 5% CO_2-grown cells than in air-grown cells, suggesting faster rates of electron transport and of NADP reduction to NADPH in CO_2-grown cells. In addition, Lonergan and Sargent note work by other researchers that shows the thylakoid organization of chloroplasts (Gergis, 1972) and the K_M (CO_2) and K_M (HCO_3^-) for photosynthetic carboxylation (Berry et al., 1976) vary depending upon the amount of CO_2 used for growth. Hence, the changes in photosynthetic rates of algal cells grown at different CO_2 concentrations could be caused by physiological factors other than, or in conjunction with, changes in carbonic anhydrase. Thus, it is difficult to identify any one controlling factor.

Experiments similar to these with algae have been carried out using higher plants. Avena sativa, a C_3 plant species, grown for four days at 80 ppm CO_2 showed twice as much carbonic anhydrase activity as plants grown at 600 ppm CO_2 (Cervigni et al., 1971). In contrast, the C_4 species

Zea mays exhibited one-third more carbonic anhydrase activity when grown at 600 ppm CO_2 as plants grown at 80 ppm CO_2. Plants of both kinds maintained at 600 ppm CO_2 for 12 hours and then transferred to normal levels (300 ppm CO_2) exhibited a return to normal enzymatic levels after 3 hours. Graham et al. (1971) performed a similar experiment. Pisum sativum and Typha sp., both C_3 plant species, and Zea mays and Sorghum bicolor, C_4 species, were grown at 0.03%, 1%, 5%, and 10% CO_2. Although carbonic anhydrase activity was somewhat reduced in both C_3 and C_4 plants at 10% CO_2, the effects were not as dramatic as those observed by Cervigni et al., or as those seen in algae. Regardless of these differences, other problems, including the physiological changes noted above for algae, are inherent in experiments involving higher plants grown at higher than normal CO_2 levels. Higher plants grown at higher than normal atmospheric concentrations of CO_2 also would experience stomatal closure which would result in lower than normal internal CO_2 levels because of internal photosynthetic depletion of CO_2 (Graham et al., 1971). Hence, the effects of higher CO_2 concentrations on carbonic anhydrase activity in higher plants can not be fully evaluated and inferences into its function in photosynthesis are hampered further.

A second method which has been employed to study the relationship between carbonic anhydrase and photosynthesis is through the use of inhibitors. Acetazolamide (Diamox), a sulphonamide, was used in early studies as a

specific inhibitor of carbonic anhydrase. Carbonic anhydrase activity was completely inhibited in four species of blue-green algae at 10^{-3} M acetazolamide (Ingle and Colman, 1975) and in two species of red algae and two species of green algae at 10^{-4} M (Bowes, 1969). Acetazolamide inhibited 50% of the activity of purified spinach leaf carbonic anhydrase at 2×10^{-5} M (Everson, 1970; 1971), of partially purified Hordeum vulgare L. leaf carbonic anhydrase at 2×10^{-6} M, partially purified Phaseolus vulgaris L. leaf carbonic anhydrase at 2.4×10^{-5} M, and partially purified Phaseolus vulgaris L. root nodule carbonic anhydrase at 3.0×10^{-6} M (Atkins, 1974). Acetazolamide at 5×10^{-5} M inhibited carbonic anhydrase from Zea mays by 85%, Amaranthus viridis by 82%, and Spinacea oleracea by 60%. The C_4 plant species were somewhat more sensitive to the inhibitor than the C_3 species (Everson, 1971). Other experiments show that acetazolamide inhibits photosynthesis as well as carbonic anhydrase. In Chlorella pyrenoidosa grown at low CO_2 levels (high carbonic anhydrase activity), photosynthesis was inhibited by more than 90% in the presence of 25 mM acetazolamide. At high CO_2 concentrations (low carbonic anhydrase) acetazolamide had no effect on photosynthesis. These results suggested that carbonic anhydrase is required for photosynthesis at low CO_2 levels, possibly to facilitate CO_2 movement into the cells, but that carbonic anhydrase is not needed for photosynthesis at high CO_2 concentrations (Graham et al., 1971). In agreement with these findings, Everson (1970)

showed that 1 mM acetazolamide inhibited photosynthesis by 50% in isolated spinach chloroplasts. The addition of 5 mM $NaHCO_3$ to the chloroplast suspension completely reversed the inhibition of photosynthesis by acetazolamide. It was postulated that this reversal was caused by increased CO_2 levels which may have eliminated the need for carbonic anhydrase. The concentration of acetazolamide, however, that was necessary to inhibit photosynthesis by 50%, was 50 times in excess of that required to inhibit 50% of the carbonic anhydrase activity. This result indicated that the inhibition of photosynthesis by acetazolamide was not caused entirely by the inhibition of carbonic anhydrase, but by some photosynthetic factor(s) or process(es) other than carbonic anhydrase. Such an alternate effect of acetazolamide on photosynthesis was demonstrated by Swader and Jacobson (1972) who showed that acetazolamide inhibits photosynthetic electron transport. Although twenty-five times more acetazolamide was required to inhibit electron transport by 50% than was required to inhibit carbonic anhydrase, the levels of acetazolamide (1 mM) that were used to inhibit photosynthesis in isolated spinach chloroplasts by Everson in the experiment described above also would have completely inhibited photosynthetic electron flow. Hence, the effects of acetazolamide on photosynthesis can not be attributed solely to inhibition of carbonic anhydrase. Lonergan and Sargent (1978) also contested the validity of experiments which used inhibitors in combination with changes

in CO_2 levels on algae as was done by Graham et al. (1971). They illustrated that the effects of acetazolamide upon cells of Euglena gracilis grown at atmospheric levels of CO_2 and at 5% CO_2 are different. Cells grown on air and treated with 10 mM acetazolamide showed a 73% reduction in photosynthesis and a 74% inhibition of DCPIP reduction, indicating that the inhibition of photosynthesis was a result of inhibition of photosynthetic electron flow rather than of carbonic anhydrase. Cells grown on 5% CO_2 showed only a 31% inhibition of DCPIP reduction, again showing that algal cells grown at different CO_2 concentrations are essentially in different physiological states. Hence, the effect of inhibitors upon these cells cannot be compared directly.

Ethoxzolamide, another sulphonamide, also has been utilized as a specific inhibitor of carbonic anhydrase. Its use presents many of the same problems as have been observed with acetazolamide. Ethoxzolamide inhibited carbonic anhydrase by 50% in isolated spinach chloroplasts at a concentration of 3.0×10^{-7} M (Jacobson et al., 1975) and 4.0×10^{-7} M (Everson, 1971). Jacobson et al., however, found that the concentration required to inhibit 20-40% of CO_2 fixation was in excess of that required to inhibit purified carbonic anhydrase, again suggesting that the inhibitor affects some process other than carbonic anhydrase function. They found, though, that unlike acetazolamide, ethoxzolamide did not inhibit photosynthetic electron flow at the concentrations required to inhibit carbonic anhydrase. This lack

of an effect, however, did not rule out the possibility that the inhibitor could affect other processes, such as chloroplast membrane permeability to CO_2 or other enzymes involved in cellular metabolism, especially in view of the data provided by Jacobson et al. (1975) that an increased HCO_3^- concentration counteracts the inhibition by ethoxzolamide and that PGA reduction is partially inhibited by ethoxzolamide. Lonergan and Sargent (1978) discovered that much higher concentrations of ethoxzolamide were necessary to inhibit carbonic anhydrase in whole cells of Euglena gracilis than was required to inhibit the carbonic anhydrase of isolated chloroplasts (Jacobson et al., 1975). The higher concentrations required to inhibit carbonic anhydrase in whole cells may be because of additional extrachloroplastic carbonic anhydrase in whole cells as opposed to only chloroplastic carbonic anhydrase in isolated chloroplasts. Ethoxzolamide at 1-5 mM was necessary to inhibit 75% of the carbonic anhydrase activity and 100% of the photosynthetic rate in whole cells of Euglena gracilis. At 5 mM ethoxzolamide photosynthetic electron flow was inhibited by 91% and 85% as measured by DCPIP reduction and methyl viologen reduction assays, respectively. In summary, the use of inhibitors for carbonic anhydrase has not been the simple panacea that was originally hoped for in resolving the functional role of carbonic anhydrase in photosynthesis.

A third experimental approach that has been used to analyze the relationship between carbonic anhydrase and photosynthesis has been through zinc nutrition studies. These studies were based upon the premise that plants placed on a zinc deficient diet would exhibit lowered levels of carbonic anhydrase. It was speculated that these lowered carbonic anhydrase levels would in turn be reflected in lowered photosynthetic capacities. Investigators have shown that plants grown on zinc deficient diets were lower than controls in the amount of zinc per leaf and that these lower leaf zinc levels were correlated with reduced carbonic anhydrase levels (Bar-Akiva and Lavon, 1969; Edwards and Mohamed, 1973; Ohki, 1976; Randall and Bouma, 1973; Wood and Sibly, 1952). The effects of lowered carbonic anhydrase activities in these plants, however, on photosynthesis were variable. Randall and Bouma (1975) found little effect of lowered carbonic anhydrase levels on net photosynthesis in spinach, except under the most severe zinc deficiencies. In the latter case, carbonic anhydrase was less than 10% that of control plants while net photosynthesis was 60-70% less, indicating that carbonic anhydrase was not the crucial factor in the determination of photosynthetic rate. In addition, when plants raised with adequate levels of zinc were transferred to low zinc or control solutions and tested for net CO_2 uptake at various ambient CO_2 levels several days after transfer, plants at lowered zinc levels and lower carbonic anhydrase levels showed no effect on net

CO_2 uptake compared to controls at ambient levels of 75-325 µl CO_2 l^{-1}. At higher CO_2 levels, carbonic anhydrase deficient plants showed a reduced ability to take up CO_2 at ambient CO_2 concentrations of 325-600 µl l^{-1}. The reverse of these results would have been expected if carbonic anhydrase were essential for facilitation of CO_2 transport to the sites of CO_2 fixation. That is, (1) at lower than normal CO_2 levels, it would have been expected that plants with lower carbonic anhydrase activity would be less efficient than the controls in taking up CO_2. But in truth, the carbonic anhydrase deficient plants were no different than controls in their ability to take up CO_2. And (2) at higher than normal CO_2 levels, it would have been expected that the CO_2 uptake abilities of carbonic anhydrase deficient plants would not be less than controls since the internal CO_2 concentrations would be higher and carbonic anhydrase would not be as essential. But, again, this relationship was not borne out as carbonic anhydrase deficient plants were less efficient in taking up CO_2 at high ambient CO_2 concentrations than controls. The experiments conducted at higher than normal CO_2 concentrations, however, did not consider the effects of stomatal closure upon CO_2 uptake.

The results of Trioli and Bassanelli (1976) agree with those outlined above. They also found no effect of lowered carbonic anhydrase levels on photosynthetic rate in zinc-deficient plants of Triticum durum. They did find,

however, that zinc-deficient plants with lowered carbonic anhydrase levels had increased rates of photorespiration as compared to plants grown at higher levels of zinc.

In contrast to the two studies discussed above, Ohki (1976) found that as zinc levels within the leaves of Gossypium hirsutum L. increased, carbonic anhydrase levels increased and, up to a leaf zinc content of 13-14 $\mu g\ g^{-1}$ dry weight, net photosynthesis increased.

No real conclusions regarding the effect of zinc nutrition and lowered carbonic anhydrase activity on photosynthesis can be made since the zinc status of the plant may affect parameters other than the carbonic anhydrase activity. For example, reduced levels of protein (Edwards and Mohamed, 1973; Ohki, 1976; Wood and Sibly, 1952) and chlorophyll (Edwards and Mohamed, 1973; Ohki, 1976; Trioli and Bassanelli, 1976) occur at lowered zinc levels and may be reflected in the slower growth rates exhibited by these plants. The activity of several enzymes, RuBPcase, glycolic oxidase, and malic dehydrogenase, also show depressed activities at lowered zinc levels in Phaseolus vulgaris L. (Edwards and Mohamed, 1973). Other researchers have noted reductions in aldolase activity (Quinlan-Watson, 1953) and auxin levels (Skoog, 1940) in zinc-deficient plants. Sibly and Wood (1952) suggested that a zinc limitation acts to depress carbonic anhydrase levels by limiting protein synthesis rather than by limiting the formation of the active carbonic anhydrase enzyme complex. This depression of

protein synthesis by a zinc deficiency also would result in reduced synthesis of other enzymes. Hence, the effects of zinc deficiencies on plants cannot be viewed as simply as an effect of reduced carbonic anhydrase activity on photosynthesis. A whole range of other processes besides carbonic anhydrase activity are affected in plants deficient in zinc and these processes also very likely affect photosynthetic metabolism.

Several theories concerning the function of carbonic anhydrase *in situ* have been formulated. Graham and Reed (1971) and Graham et al. (1971) suggested several roles for the enzyme related to CO_2 availability during photosynthesis. One hypothesis suggests that carbonic anhydrase acts as a permease to facilitate the transport of CO_2 across the chloroplast envelope. Enns (1967) and Broun et al. (1970) demonstrated that carbonic anhydrase could enhance the diffusion of inorganic carbon across artificial membranes. In order for this system to operate *in vivo*, carbonic anhydrase would have to be located in the chloroplast membrane. The bulk of carbonic anhydrase activity, however, resides in the stroma. Very little activity is associated with the chloroplast membrane (Everson, 1970; Jacobson et al., 1975; Poincelot, 1972a). Hence, carbonic anhydrase probably has little to do with the transport of carbon across the chloroplast membrane.

A second theory explores the possibility that carbonic anhydrase is physically associated with RuBPcase and

that it serves to directly supply the carboxylase with CO_2. Graham et al. (1971), however, found no such association between carbonic anhydrase and fraction I protein in Chlamydomonas or in leaves of higher plants using gel electrophoresis. (Fraction I protein consists essentially of RuBPcase (Zelitch, 1971).) These results, however, do not eliminate the possibility of an in vivo interaction of the two enzymes. A theoretical analysis using the K_M (CO_2) values of the two enzymes reveals that such an interaction is unlikely (Jacobson et al., 1975). A physical interaction of the two enzymes would assume that RuBPcase has a lower affinity for CO_2 than carbonic anhydrase has for CO_2 and that the affinity of RuBPcase for a carbonic anhydrase-CO_2 complex is greater than its affinity for free CO_2. Such an interaction is unlikely since RuBPcase has a higher affinity for CO_2 (K_M = 450-560μM) (Bahr and Jensen, 1974) than carbonic anhydrase has for the hydration of CO_2 (K_M = 29.9mM) (Jacobson et al., 1975). Hence, it is unlikely that carbonic anhydrase aids photosynthesis by first binding CO_2 and then transferring it to RuBPcase.

An alternative suggestion is that carbonic anhydrase does not necessarily bind CO_2 for direct transfer to RuBPcase, but that it catalyzes the formation of CO_2 within the chloroplast. As CO_2 is formed, it may be immediately assimilated by RuBPcase. Since the pH within the chloroplast is 7.7-8.4 (Moyse, 1975), HCO_3^- is more abundant than CO_2.

At pH 7.9, free CO_2 represents less than 1% of the total inorganic carbon within the chloroplast (Buchanan and Schürmann, 1973). Since CO_2 is the substrate for carboxylation by RuBPcase (Cooper et al., 1969), then some mechanism, which could be explained by the action of carbonic anhydrase, must push the equilibrium toward CO_2. As CO_2 is produced by the action of carbonic anhydrase, this CO_2 may be quickly taken up by the carboxylase so that the overall free dissolved CO_2 concentration may not change and the pH would remain elevated. In addition, the K_M of RuPBcase for CO_2 is lower in intact chloroplasts than in the isolated form (Bahr and Jensen, 1974), suggesting that some factor, such as carbonic anhydrase, operates within the chloroplast to lower the K_M of RuBPcase.

The idea that perhaps carbonic anhydrase is essential for making CO_2 available to the sites of CO_2 fixation in the chloroplast was originally supported by the difference observed in the levels of carbonic anhydrase between C_3 and C_4 plants (Everson and Slack, 1968). In this study, it was observed that C_3 plants had much higher levels of carbonic anhydrase than C_4 plants. It was postulated that carbonic anhydrase was essential in C_3 plants to facilitate the transport of CO_2 to the sites of fixation and to concentrate CO_2 at those sites. It was believed that perhaps C_4 plants did not require the carbonic anhydrase levels observed in C_3 plants because the dicarboxylic acid cycle and the enzyme PEPcase performed the same functional role of transport and

concentration of CO_2 in C_4 plants as was performed by carbonic anhydrase in C_3 plants. The results of Poincelot (1972b), however, showing levels of carbonic anhydrase in a C_4 plant equivalent to C_3 plants raised a question as to the validity of this concept. This concept still may apply as Poincelot showed that the carbonic anhydrase activity in C_4 plants is restricted to the mesophyll cells and that little carbonic anhydrase activity is associated with the bundle sheath cells. Hence, the dicarboxylic acid cycle which operates to shuttle CO_2 from the mesophyll to the bundle sheath, may still be acting in lieu of carbonic anhydrase so that the enzyme is not required in the bundle sheath cells.

Graham and Reed (1971) suggested another possible role for carbonic anhydrase which dealt not with CO_2, but with the ability of the enzyme to regulate H^+ concentrations. According to Graham and Reed, this ability could have two foreseeable roles: (1) to rapidly generate the large number of protons that are necessary to establish and maintain a proton gradient across the thylakoid membranes for photophosphorylation, and (2) to rapidly buffer the pH changes associated with photosynthesis. The experiments performed to support these theories, however, either employed the use of inhibitors or used algal cells grown at different CO_2 concentrations (Everson, 1971; Graham et al., 1971; Rybová and Slavíková, 1973). As discussed previously, neither of these methods is reliable in isolating the actual role of

carbonic anhydrase in photosynthesis. In addition, later research by Graham et al. (1974) did not provide consistent, reproducible results with regard to the enzyme functioning in either of these two roles.

Other experiments have suggested a relationship between carbonic anhydrase activity and ion transport. Findenegg (1974) studied the relationship of carbonic anhydrase to Cl^- and HCO_3^- fluxes in air-adapted and CO_2-adapted (1.5% CO_2) cells of Scenedesmus obliquus. Air-adapted cells had 20 times greater carbonic anhydrase activity than CO_2-adapted cells and were shown to be able to photosynthesize efficiently at high HCO_3^- concentrations at pH 9.2. At pH 5.8, where CO_2 dominates over HCO_3^-, air-adapted cells took up Cl^- in place of HCO_3^-. Carbon dioxide-adapted cells, however, were unable to take up Cl^- at pH 5.8 and were unable to utilize HCO_3^- for photosynthesis at pH 9.2. Therefore, it appeared that carbonic anhydrase was required for the uptake of HCO_3^- at high pH values and for Cl^- uptake at low pH values. Neither of these transport processes occurred in the absence of carbonic anhydrase. These experiments are again difficult to interpret in terms of the actual role of carbonic anhydrase in plant cells. As pointed out above, growing algae at varying CO_2 concentrations affects processes other than carbonic anhydrase and some of these may affect HCO_3^- and Cl^- ion transport.

Work by Rybová and Slavíková (1973) also investigated the effect of carbonic anhydrase levels on ion transport in

Hydrodictyon reticulatum using the inhibitor hydrochlorothiazide. Their results concerning Cl^- transport were consistent with Findenegg's data, but, again, the interpretation of studies involving the use of an inhibitor is problematic.

It also has been suggested that the presence of carbonic anhydrase in aquatic plants may facilitate the utilization of HCO_3^- as a source of carbon for photosynthesis at high pH values in an aquatic environment. Some aquatic plants, including both algae and higher aquatic vascular plants, are able to use HCO_3^- in addition to free dissolved CO_2 as a source of carbon for photosynthesis. Other plants are able to use only CO_2 (Hutchinson, 1975). These facts led to the suggestion that the ability of some aquatic plants to use HCO_3^- is governed by the presence of carbonic anhydrase. The existence of carbonic anhydrase in these plants would supposedly allow for the rapid conversion of HCO_3^- to CO_2. This CO_2 then could be photosynthetically assimilated. (Raven, 1970; Steemann Nielsen and Kristiasen, 1947). Studies considering this possibility, however, have not confirmed this concept. Steemann Nielsen and Kristiansen (1947) compared the carbonic anhydrase levels of two aquatic plant species: Elodea canadensis, a vascular aquatic plant, capable of using HCO_3^- as a source of carbon for photosynthesis, and Fontinalis dalicarlica, an aquatic moss able to use only free dissolved CO_2. They found no differences in the carbonic anhydrase levels of these two plants and concluded that carbonic anhydrase was not involved in the utilization of HCO_3^-. But, the

conditions, such as pH and HCO_3^- concentrations, under which these plants had been growing prior to assay for the enzyme were not reported. Since the enzyme appears to be inducible according to studies done by Nelson et al. (1969) and others, it is possible that the conditions under which E. canadensis had been growing may not have been proper for induction of enzymatic synthesis resulting in carbonic anhydrase levels in E. canadensis that were no higher than those found in F. dalicarlica. Hence, the use of proper controls and careful monitoring of pH and HCO_3^- concentration would have added valuable information to this study. Secondly, this study would have been more meaningful if the two plants that were compared had been from the same hierarchical level in the plant kingdom, i.e., if both higher vascular plants were used rather than a vascular plant and a moss.

Österlind (1950) performed a similar study using two species of green algae. He also found no differences in carbonic anhydrase activity between Chlorella pyrenoidosa, a species able to use only free dissolved CO_2, and Scenedesmus quadricauda, a species able to use HCO_3^-. However, this study also failed to run adequate controls and to look at the enzymatic activity in relation to HCO_3^- concentration.

Carbonic anhydrase levels also have been implicated in the excretion of glycolate by Coccochloris peniocystis (Cyanophyta), but some of the same problems exist here as those discussed previously. Ingle and Colman (1974) demonstrated that when cells of this alga were grown in 5%

CO_2 (carbonic anhydrase levels low) and then transferred to growth in air, glycolate was excreted at a linearly decreasing rate while carbonic anhydrase levels rose. The researchers proposed that at low CO_2 concentrations, glycolate formation was enhanced by increased activity of the oxygenase function of RuBP carboxylase/oxygenase. The oxygenase would have been stimulated by the higher O_2 tensions that would have resulted when the algae were transferred from a high CO_2 to a low CO_2 concentration. The researchers did not define a role for carbonic anhydrase in relation to glycolate formation. An attempt to do so would be difficult in view of the different physiological states of cells grown at different CO_2 concentrations (Lonergan and Sargent, 1978).

Carbonic anhydrase levels respond to varying light intensities. Angiosperms, both C_3 and C_4 plants, grown at high light intensities showed higher levels of carbonic anhydrase activity than plants grown at low light intensities (Everson, 1971). Plants also showed reduced carbonic anhydrase levels when placed in darkness for 4 to 5 days (Everson, 1971; Waygood and Clendenning, 1950). These results suggest that at least a portion of the synthesis of carbonic anhydrase is dependent upon light. In addition, Waygood and Clendenning (1950) observed that mutant albino leaves of barley have 75% less carbonic anhydrase than normal leaves and that the white portion of variegated Tradescantia leaves had 50-60% lower carbonic anhydrase than the green portion. These facts raise

questions as to the dependence of carbonic anhydrase synthesis on not only light, but also upon the development of chlorophyll in the plant.

In summary, carbonic anhydrase has been implicated in several processes in green plants: in CO_2 transport and fixation, and in ion fluxes. A great deal of research has been done on the enzyme, but progress in delineating its exact function has been confronted by many technical obstacles. Hence, although the enzyme appears to be involved in photosynthetic metabolism, its exact role(s) have yet to be established.

OBJECTIVES

The main objective of this thesis project was to employ a comparative approach to the study of the enzyme carbonic anhydrase by examining its occurrence in a variety of aquatic macrophytes. Secondarily, an attempt was made to determine how carbonic anhydrase levels are regulated by looking at the internal lacunar CO_2 concentrations of these plants in relation to their respective carbonic anhydrase levels.

A comparative approach has been utilized in the past by other investigators in an effort to determine the actual function of carbonic anhydrase in plants (i.e., the comparative studies of C_3 and C_4 plants by Everson and Slack, 1968, and Poincelot, 1972b). Similarly, at the onset of this study, it was believed that a comparative approach using aquatic macrophytes could lead to some insight into the function of carbonic anhydrase in plants. It was especially felt that these plants could prove to be interesting because of the wide range of habitats in which they occur. The fact that these plants grow as either emergent, floating, or submersed forms means that they exhibit a wide range of anatomical and morphological characteristics that provide

special adaptive features to their respective habitats. In addition to the morphological and anatomical adaptations which these plants possess, special biochemical and physiological adaptations also are required. Biochemical and physiological adaptations may particularly influence the photosynthetic metabolism of these plants, a fact that may be reflected in the gross differences in productivity observed between aquatic plants from different habitats (Wetzel, 1975). In addition, because there are differences in the forms of inorganic carbon available to these plants from different habitats implies that perhaps differences in the carbonic anhydrase levels of these plants could be expected. Submersed plants live in a very different environment from which they must obtain CO_2 than is found for emergent plants. Submersed plants may acquire CO_2 either as dissolved CO_2 or HCO_3^- from an aqueous medium whereas emergent plants obtain gaseous CO_2 directly from the atmosphere. This difference in how these plants must acquire CO_2 may also lead to differences in levels of the enzyme responsible for handling CO_2: namely, carbonic anhydrase.

Aquatic macrophytes were also used in this study as a means to investigate a possible method by which carbonic anhydrase levels are regulated in plants. Because of the internal lacunar system which these plants possess, CO_2 gas of respiratory and photorespiratory origin accumulates within the plant tissue and hence allows for the photosynthetic re-fixation of this CO_2 (Søndergaard and Wetzel, 1979). In

addition, the slow diffusion of gases in water could limit the amount of CO_2 diffusing out of the lacunae. This combination of factors could lead to CO_2 concentrations that are higher than atmospheric levels within these plants. It was postulated at the beginning of this study that differences in the level of CO_2 within aquatic macrophytes from different habitats could partly explain the differences observed in the levels of carbonic anhydrase in these plants. Carbonic anhydrase levels have been shown to change in algae in response to various CO_2 concentrations (Graham et al., 1971; Ingle and Colman, 1975; Nelson et al., 1969). High internal CO_2 concentrations were considered in this study to be a possible factor in the low carbonic anhydrase levels which had been observed in several submersed aquatic macrophytes (Van et al., 1976). In contrast, lower internal CO_2 concentrations in emergent aquatic macrophytes may result in the increased carbonic anhydrase levels observed in these plants. Typha latifolia has been reported to possess carbonic anhydrase levels comparable to terrestrial plants (Atkins et al., 1972b). Although T. latifolia also possesses an internal lacunar gas system, its aerial growth form probably results in a more rapid and complete equilibration of the lacunar gases with the surrounding atmosphere than is possible for submersed plants. This equilibration may be facilitated by wide-open stomata since emergent aquatic macrophytes are generally rooted in saturated soil and

hence probably do not need to be concerned with excessive transpiratory water loss from its tissues. Consequently, the hypothesis was formulated for this study that possible high internal CO_2 concentrations in submersed aquatic macrophytes could lead to an inhibition of enzymatic synthesis of carbonic anhydrase and the resulting lower carbonic anhydrase activities. It follows then that lower internal CO_2 concentrations in emergent aquatic macrophytes could result in less repression of carbonic anhydrase synthesis and subsequent higher carbonic anhydrase levels.

MATERIALS AND METHODS

Plant Material.--For the bulk of this study in which carbonic anhydrase levels and internal CO_2 levels were compared in a variety of aquatic macrophytes, plant material was collected fresh from four lakes: Duck Lake, Lawrence Lake, Three Lakes, and Wintergreen Lake. Duck Lake is a softwater lake and the latter three are hardwater lakes. The exact location of these lakes is given in Table 1. Plant material was collected from mid-May through late June 1978. Only healthy, vigorously growing plants were used for the enzymatic and protein assays and internal CO_2 gas analyses. Plants were thoroughly rinsed free of sediment, epiphytes, and calcium carbonate before use. A list of the plants collected and the location of collection is given in Table 1.

Several species included in this study are not typical of Michigan's temperate zone, but were collected in Florida. These plants were transported to Michigan on ice in a cooler to reduce respiration and decomposition. Upon arrival in Michigan the plants were immediately rinsed, planted in clean silica sand in large tubs, and well water added. The water was continuously aerated and the water

Table 1

List of plant species and collection sites.

Plant Species	Collection Site
Submersed	
Ceratophyllum demersum L.	Three Lakes[1]
Chara sp.	Three Lakes
Elodea canadensis Michx.	Three Lakes
Lemna trisulca L.	Roadside Ditch
Myriophyllum heterophyllum Michx.	Three Lakes
Potamogeton crispus L.	Three Lakes
Potamogeton foliosus Raf.	Three Lakes
Potamogeton natans L.	Three Lakes
Potamogeton pectinatus L.	Lawrence Lake[2]
Potamogeton praelongus Wulf.	Lawrence Lake
Scirpus subterminalis Torr.	Lawrence Lake
Utricularia sp.	Duck Lake[3]
Vallisneria americana Michx.	Three Lakes
Floating-leaved	
Brasenia Schreberi Gmel.	Duck Lake
Nuphar variegatum Engelm.	Lawrence Lake
Nymphaea tuberosa Paine	Lawrence Lake
Free-floating	
Eichhornia crassipes (Mart.) Solms	Florida
Lemna minor L.	Wintergreen Lake[4]
Wolffia columbiana Karst.	Wintergreen Lake
Emergent	
Equisetum fluvatile L.	Three Lakes
Hydrocotyle ranunculoides L. F.	Florida
Myriophyllum brasiliense Camb.	Florida
Nasturtium officinale R. Br.	Greenhouse[5]
Peltandra virginica L.	Three Lakes
Pontederia cordata L.	Three Lakes
Scirpus acutus Muhl.	Greenhouse
Typha latifolia L.	Three Lakes

[1]Sec. 25, T.1S., R.10W., Kalamazoo Co., Michigan.

[2]Sec. 27, T.1N., R.9W., Barry Co., Michigan.

[3]Sec. 5, T.1S., R.9W., Kalamazoo Co., Michigan.

[4]Sec. 8, T.1S., R.9W., Kalamazoo Co., Michigan.

[5]Plants were grown in a greenhouse. *N. officinale* was propagated from plants originally collected from Lawrence Lake. *S. acutus* was grown from rhizomes collected from Lawrence Lake.

temperature maintained at about 20°C. In general, these plants were in fairly good condition and only the best specimens were used for the assays.

Plants which were used in the preliminary phases of this research (for carbonic anhydrase and protein assays), were either collected fresh from the field and maintained in a greenhouse, or were purchased from a local aquarium shop and maintained in aquaria in growth chambers. Megalodonta Beckii, Potamogeton praelongus, and Vallisneria americana plants were collected fresh from the field and planted in clean silica sand in large tubs with well water. The water was continuously aerated and the temperature averaged 20°c. Dormant, underground rhizomes of Peltandra virginica were collected and treated similarly, the foliage being collected and used for assays as the plants grew. Nasturtium officinale plants were also collected, along with the organic mud in which they were growing, and planted in pans. These plants were allowed to propagate and to grow emergent in saturated soil. Cabomba sp. and Elodea sp. plants, which appeared to be tropical in origin, were purchased and placed in aquaria filled with well water.

Preparation of Plant Material.--Most aquatic plant species were extremely difficult to grind and normal grinding techniques were not sufficient to completely macerate the plant tissue. This was especially true for submersed plant species in which relatively large quantities

of plant material (2-4 g fresh weight) were required in order to detect carbonic anhydrase activity. Floating-leaved and emergent plants generally did not require as much plant material (approximately 0.5 g fresh weight), but were also very tough. Chopping the plant material with razor blades and then grinding the tissue in buffer with a teflon pestle driven by an electric motor did not serve to adequately macerate the plant tissue. Maceration of the plant tissue was best accomplished by grinding the chopped plant material in liquid N_2 using a porcelain mortar and pestle. The addition of liquid N_2 to the plant material caused the tissue to become very brittle and so permitted easier grinding. The resulting finely ground powder was immediately weighed into three sub-samples: one sample for a carbonic anhydrase assay; a second sample for a protein determination; and a third sample for a determination of fresh weight to dry weight ratio. Dry weights were determined after drying the plant powder in pre-dried, tared crucibles for at least 24 hours at 105°C.

The carbonic anhydrase and protein sub-samples were immediately quantitatively transferred to a grinding vessel and buffer added. These samples were then again macerated with a teflon pestle driven by an electric motor to insure more complete homogenization of the plant tissue, which was considered complete when chunks of plant tissue were no longer visible. This crude extract was then filtered

through 4 layers of cheesecloth to remove particulate material and the resulting plant extract used for the enzymatic and protein assays.

For preparation of the plant extract for the carbonic anhydrase assay, a buffer consisting of 0.10 M Tris, 0.010 M 2-mercaptoethanol, and 0.001 M Na_2-EDTA adjusted to a pH of 8.3 with HCl was used (Nelson et al., 1969). Samples were held in an ice-bath during the final homogenization step in order to help stabilize the enzyme. Protein samples were ground at room temperature in a 0.05 M KH_2PO_4 buffer adjusted to pH 8.3 with NaOH.

Carbonic Anhydrase Assay.--An electrometric method was used to assay for carbonic anhydrase. This method consisted of measuring the rate of hydration of CO_2 ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$) over time by the reduction in pH. Carbon dioxide-saturated water was used as the substrate and was prepared by passing purified CO_2 gas through about 800 ml of glass distilled water at 1°C for at least one hour before use. The water was contained within a 1-liter Erlenmeyer flask which had an opening that was sealed with a serum bottle stopper at the base of the flask. CO_2-saturated water was withdrawn through the serum stopper using a 5-ml glass syringe fitted with a cannula. During withdrawal of the CO_2-saturated water, the flask and syringe were tipped slightly to force bubbles forming in the water into the syringe-cannula junction. These bubbles were then ejected

and fresh CO_2-saturated water taken-up to replace the evacuated water. This procedure allowed for the elimination of bubbles and gave a bubble-free uniform volume of CO_2-saturated water.

To perform the assay, 1 ml of plant extract, prepared according to the above procedure, and 5 ml of 0.025 M Veronal buffer (sodium barbital-HCl) at pH 8.2 were pipetted into a 10-ml round-bottomed reaction flask. This reaction flask was placed in an ice-bath to maintain the plant extract-buffer mixture at about 1°C. The assay was run at this temperature in order to slow the reaction rate sufficiently so that it could be measured. A combination pH electrode, connected to a Coleman Model 38A pH meter, was lowered into the plant extract-buffer mixture and allowed to equilibrate before the assay. In general, the pH was between 8.3 and 8.45 before initiation of the assay. The assay was started by rapidly injecting 5 ml of CO_2-saturated water into the plant extract-buffer mixture. The time for the pH to drop from 8.0 to 7.0 was measured with a stop-watch. A blank, consisting of 1 ml of 0.10 M Tris, 0.010 M 2-mercaptoethanol, and 0.001 M Na_2-EDTA buffer at pH 8.3 and 5 ml of 0.025 M Veronal buffer at pH 8.2 was assayed as above.

Enzyme units were calculated according to Wilbur and Anderson (1968) using the formula:

$$E.U. = 10[(t_b/t_e)-1]/\text{mg protein (or dry weight)}$$

where t_b = non-enzymatic time in seconds using buffer and

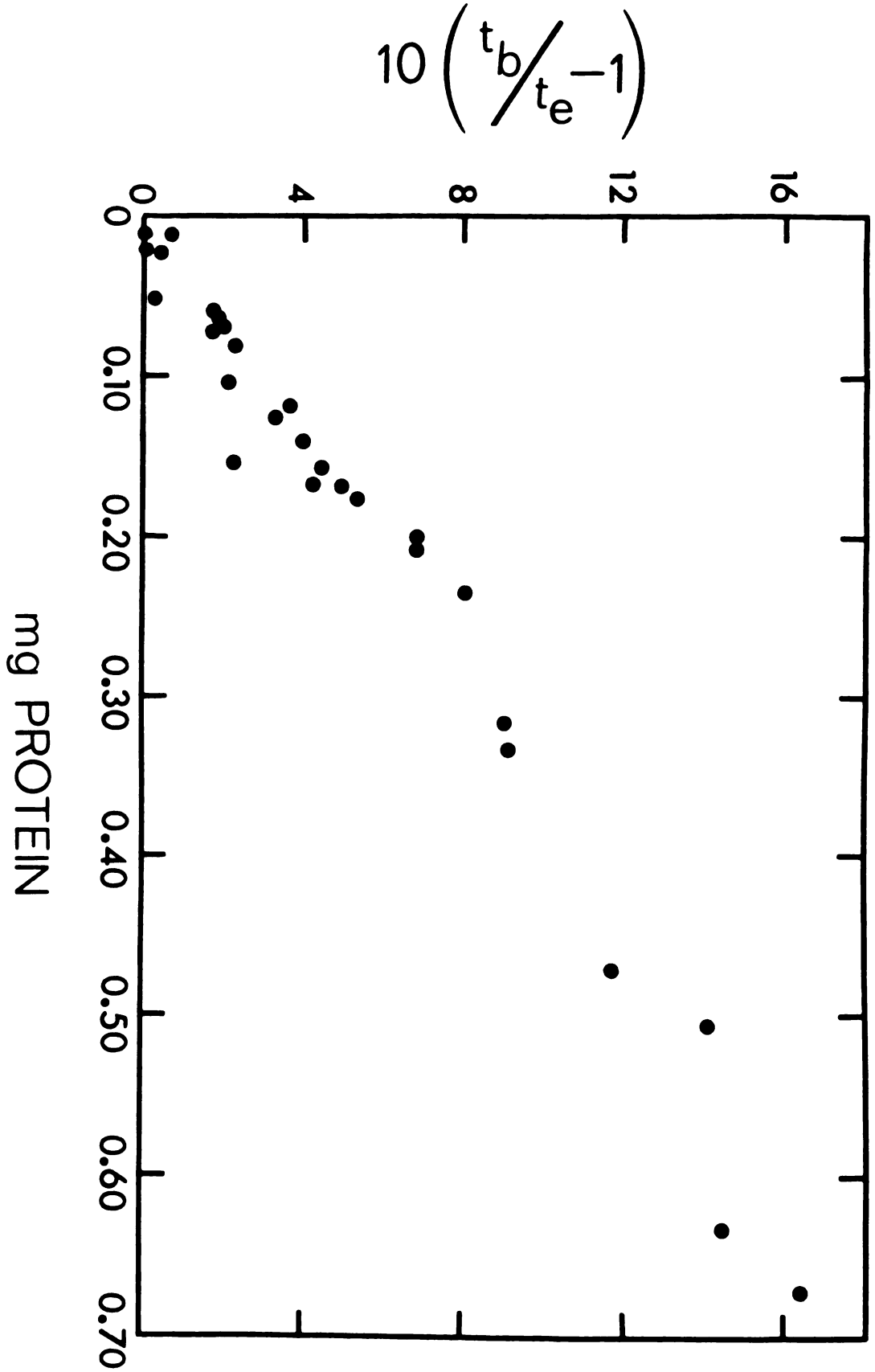
t_e = enzymatic time in seconds using plant extract

Linearity of the enzymatic assay was determined by using a plant extract prepared from Nasturtium officinale. Since N. officinale leaves are easily macerated and have relatively high carbonic anhydrase activities, they were not ground in liquid N_2 but were placed directly into buffer and homogenized. The carbonic anhydrase assay then was performed using varying volumes of the plant extract. These volumes were converted to mg of protein (see below for protein assay) and plotted against $10[(t_b/t_e)-1]$ values (Fig. 1). Linearity of the assay appears to lie from a $10[(t_b/t_e)-1]$ value of 0 to 8. In performing the assays on aquatic plants, only t_e values within the range of 36.14 sec. and 65.06 sec., using an average t_b value of 65.06 sec., were accepted. This range of t_e values lies within the ranges stated above for $10[(t_b/t_e)-1]$. Samples where 1 ml of plant extract gave t_e values less than 36.14 sec. were diluted with the buffer to give values within the range stated above.

To determine whether liquid N_2 affects carbonic anhydrase activity, a sample treated with liquid N_2 was compared to an untreated control. Leaves of Nasturtium officinale were used. Carbonic anhydrase activity in a plant extract prepared from leaves which were homogenized directly in buffer was compared to a plant extract from which the leaves had been treated with liquid N_2 prior to

FIGURE 1

Linearity of carbonic anhydrase assay using leaves of *Nasturtium officinale*


$10\left(t_b/t_e - 1\right)$
0
4
8
12
16
0
0.10
0.20
0.30
0.40
0.50
0.60
0.70
mg PROTEIN

homogenization. Fresh leaves had an E.U. (mg^{-1} protein) of 40.44 ± 1.632 (SE) versus 42.08 ± 3.576 (SE) for leaves which had been frozen in liquid N_2, with five replicates for each treatment. These results showed no significant difference between fresh leaves and leaves which had been treated with liquid N_2.

Initial assays revealed extremely low carbonic anhydrase levels in several submersed aquatic macrophytes. The existence of low levels of enzymatic activity raised the question of the possibility that an inhibitor of carbonic anhydrase could be present within the plant cells which could be released upon maceration of the plant tissue and subsequently inhibit the enzyme. To test this possibility, plant extracts from three different submersed aquatic plants were combined with two different carbonic anhydrase internal standards and assayed for carbonic anhydrase activity. The two carbonic anhydrase internal standards tested were purified bovine carbonic anhydrase (Sigma Chemical Co.) and a plant extract prepared from Nasturtium officinale. A buffer consisting of 0.010 M HEPES, 0.005 M 2-mercaptoethanol, and 0.001 M Na_2-EDTA, pH 8.5, was used to prepare both the plant extracts and the bovine carbonic anhydrase solution. The combined aquatic plant extracts and carbonic anhydrase internal standards were compared with a control consisting of the carbonic anhydrase internal standard alone and with a control consisting of the aquatic plant extract alone (Table 2). For the purposes of this experiment, it was

Table 2

Tests for naturally occurring carbonic anhydrase inhibitors in plant extracts from three submersed aquatic macrophytes using two different carbonic anhydrase internal standards.
(a) Purified bovine carbonic anhydrase as the carbonic anhydrase internal standard (B-CA)
(b) Plant extract of Nasturtium officinale as the carbonic anhydrase internal standard (WC-CA)

(a) Treatment	t_e ± SE
Control (B-CA + buffer)	7.14 ± 0.300*
Control (plant extract of Megalodonta Beckii)	34.31**
Control (plant extract of Potamogeton praelongus)	33.10
B-CA + plant extract of Megalodonta Beckii	12.26**
B-CA + plant extract of Potamogeton praelongus	9.11**
B-CA + plant extract of Vallisneria americana	10.58 ± 0.406*
t_b = 34.74 ± 2.043 ($\bar{x}$ ± SE; n = 6)	

(b) Treatment	t_e
Control (WC-CA + buffer)	10.12**
Control (plant extract of Megalodonta Beckii)	39.40
Control (plant extract of Potamogeton praelongus)	37.30
Control (plant extract of Vallisneria americana)	38.85
W-CA + plant extract of Megalodonta Beckii	10.81**
W-CA + plant extract of Potamogeton praelongus	11.11**
W-CA + plant extract of Vallisneria americana	11.35
t_b = 37.76 ± 0.838 ($\bar{x}$ ± SE; n = 3)	

t_e = enzymatic time in seconds.

t_b = non-enzymatic time in seconds.

A low t_e value denotes a high carbonic anhydrase activity whereas a high t_e value approaching the value of t_b denotes a low carbonic anhydrase activity.

* t_e value is the mean of three replicates.

** t_e value is the mean of two replicates.

adequate to compare the relative effects of the submersed aquatic plant extracts on the carbonic anhydrase activity of the internal standards by using t_e values rather than actual enzyme units such that low t_e values denoted a high carbonic anhydrase activity and high t_e values approaching the value of t_b denoted a low carbonic anhydrase activity. The t_e values for carbonic anhydrase activity in both the bovine carbonic anhydrase solution and N. officinale plant extract were not significantly altered upon addition of the plant extracts from each of the three submersed aquatic plants.

Protein Assay.--Lowry's method of protein determination was used as outlined by Brewer et al. (1974) using the plant extract prepared for protein analysis as described earlier. To corroborate the use of the Lowry method for the quantification of protein form aquatic plants, the Lowry method and the Biuret method (according to Layne, 1957) for protein determination were compared using several different aquatic plant extracts (Table 3). The Biuret and Lowry methods for the determination of protein compare fairly well. Since Lowry's method of protein determination is more sensitive than the Biuret, the Lowry method was chosen for use in this study (lower limit of detectability is 20 µg/1 ml for the Lowry versus 1 mg/1 ml for the Biuret method).

Analysis of Internal CO_2 Concentrations in the Lacunae of Aquatic Macrophytes.--Previous techniques used for the analysis of the internal lacunar gases of aquatic

Table 3

Comparison of the Lowry and Biuret methods for the determination of protein. (Three separate samples from each plant species were compared.)

Plant Species		mg protein/ml Biuret	Lowry	B:L*	$\bar{x}$ B:L ± SE
Nasturtium officinale	1	1.96	1.12	1.75	
	2	2.16	1.14	1.89	1.82 ± 0.072
	3	2.64	1.44	1.83	
Peltandra virginica	1	6.01	2.52	2.83	
	2	6.99	3.25	2.15	2.00 ± 0.468
	3	5.94	4.00	1.48	
Cabomba sp.	1	10.90	7.52	1.44	
	2	5.11	4.77	1.07	1.19 ± 0.216
	3	4.82	4.49	1.07	
Elodea sp.	1	2.03	0.98	2.07	
	2	1.50	0.82	1.82	1.81 ± 0.265
	3	1.81	1.17	1.54	

*B:L = Biuret:Lowry.

macrophytes have involved the use of vacuum extraction techniques. These techniques are cumbersome to perform, difficult to use on a routine basis, and may not completely extract the gases from the plant. A new technique for the extraction of internal gases from aquatic macrophytes was developed. This technique is theoretically sound and allows for simple and rapid extraction of gases from aquatic plants.

This method essentially consists of placing aquatic plant material into a serum bottle which had been flushed with nitrogen and subsequently freezing the sample. Upon freezing, breakage of the plant cell walls occurs and the gases within the lacunar spaces are released. Analysis of the gases within the serum bottle gives the gaseous content of the internal lacunae of the plant. A more detailed description of the technique follows. A 30-ml serum bottle is held in an inverted position and flushed with purified nitrogen gas for 3 minutes. The aquatic plant material is quickly added, a serum bottle stopper put into place, and an aluminum seal cap crimped over the serum bottle stopper. The bottle with the plant material in it is then reflushed with nitrogen gas for 75 seconds and a slight positive pressure added by allowing the nitrogen gas to flow into the bottle with no outlet for 15 seconds. This addition of a positive gas pressure within the bottle allows for contraction of the gases when the bottle and plant material is frozen. After addition of the positive gas pressure to the

serum bottle, the bottle with the plant material inside is placed in an ultra-freezer at -60°C for at least one hour. The samples are then removed from the ultra-freezer, immediately placed on ice, and allowed to equilibrate at 0°C. The samples are kept on ice in order to eliminate the possibility of CO_2 being produced by respiration or decomposition of the plant tissue. (Respiration is, for all practical purposes, zero at 0°C.) A 1 ml gas sample is taken from the bottle with a Becton-Dickinson glasspak syringe fitted with a 20 gauge long hypodermic needle and the gas sample immediately injected into a Beckman Model 865 Infrared CO_2 analyzer. The area under the peak produced by the CO_2 present in the sample was automatically integrated. Control serum bottles, in which no plant material was added, were run in order to verify that all atmospheric gases were flushed out of the bottle with the nitrogen gas and to check for any leakage of atmospheric gases into the serum bottles which could have occurred upon freezing and thawing of the sample. Standard curves were made by using purified CO_2 gas and injecting several different volumes of the purified gas into the infrared CO_2 analyzer with a 5-µl Hamilton syringe. Since the gas samples taken from the serum bottles were at 0°C and since the standard curve was made with CO_2 gas at room temperature, the standard curve of CO_2 volumes were converted from the volume at room temperature to the volume at 0°C using the Ideal Gas Law ($\frac{V_1}{V_2} = \frac{T_1}{T_2}$). Using this standard curve at 0°C, the values for the

concentration of CO_2 in 1 ml of gas sample from each serum bottle was determined. In order to express the data on a dry weight basis, the plant tissue was dried at 105°C for 24 hours and weighed. To determine the total volume of CO_2 gas present in the bottle which had been released by the amount of measured dry plant tissue, the volume of each bottle used for gas measurements was determined. This calibration was done by weighing each bottle, filling it with water, and then weighing the bottle filled with water. By knowing the temperature of the water and the density of the water at that temperature, the actual volume of the bottle was calculated. From the measurement of CO_2 volume within the bottle which had been released from a specific quantity of plant material, µl CO_2 per gram dry weight of plant material was computed.

All plants were thoroughly cleaned of adhering soil and calcium carbonate before use. In almost all of the submersed aquatic plants, only leaves were used for the internal CO_2 analyses. For Myriophyllum heterophyllum, Ceratophyllum demersum, and Elodea canadensis, however, the stem with attached leaves was used for analyses. In the case of Lemna minor, Lemna trisulca, and Wolffia columbiana, the whole plant consisting of "leaves" with attached roots was used. For Nuphar variegatum, Nymphaea tuberosa, Eichhornia crassipes, Typha latifolia, Scirpus acutus,

Peltandra virginica, Pontederia cordata, and Equisetum fluviatile, only portions of leaves were used. Because of this, these latter plant species required special methods in order to prevent the loss of gases from the leaf segment used for analysis. For these species, whole leaves or portions of leaves were submersed in a saturated $(NH_4)_2SO_4$ solution which eliminated the possible loss of gases from the lacunae into the water. A leaf segment was then carefully sliced from this submersed leaf with a razor blade. The resulting leaf segment was then quickly placed into the N_2-flushed serum bottle. Whole leaves of Hydrocotyle ranunculoides and the floating-leaves of Potamogeton natans were used for CO_2 gas analysis. Myriophyllum brasiliense was sampled the same as M. heterophyllum.

The differences stated above for the methods of sampling aquatic plant material for CO_2 gas analyses could very well result in differences in the amount of CO_2 per gram of plant material observed solely because of differences in sampling technique. The inclusion of stems in the sampled plant material for some plant species could have biased CO_2 measurements in the direction of a greater observed concentration because of possible accumulation of CO_2 in the stems. Also, it is not known how much lacunar gas could have been lost upon cutting of the leaves or upon transfer of normally submersed leaves to air. Lacunar gases could very easily diffuse from the cut edges of a leaf and the lack of a cuticle by submersed leaves would probably mean that

diffusion of lacunar gases into the air would be very rapid as soon as they were removed from the water. Hence, the results of these analyses should probably be looked at in a qualitative way even though attempts were made to make the technique quantitative.

RESULTS AND DISCUSSION

Results of assays for carbonic anhydrase and the internal CO_2 concentrations for all plants examined are shown in Table 4. Carbonic anhydrase activities are expressed on both protein content and dry weight. Internal CO_2 concentrations are based on dry weight of the tissue. When collected, these plants were healthy, growing vigorously, and near their seasonal peak. This fact must be considered in examination of the data as the carbonic anhydrase and internal CO_2 concentration data are representative of mature plants and may not be representative of young plants or flowering and senescent plants at other times during the growing season.

Figure 2 shows the relative carbonic anhydrase activities of different plants in the habitat gradient going from a submersed to floating to emergent growth form. In general, carbonic anhydrase activities increased across this habitat gradient such that activities were low in submersed plants and higher in emergent plants. Leaves of floating-leaved and free-floating plants had intermediate activities. Across the habitat gradient, carbonic anhydrase levels did not appear to be related to the hierarchical position of

Table 4

Carbonic anhydrase activities and internal lacunar CO_2 concentrations for all plants surveyed. ($\bar{x}$ ± SE; number of replicates in parentheses.)

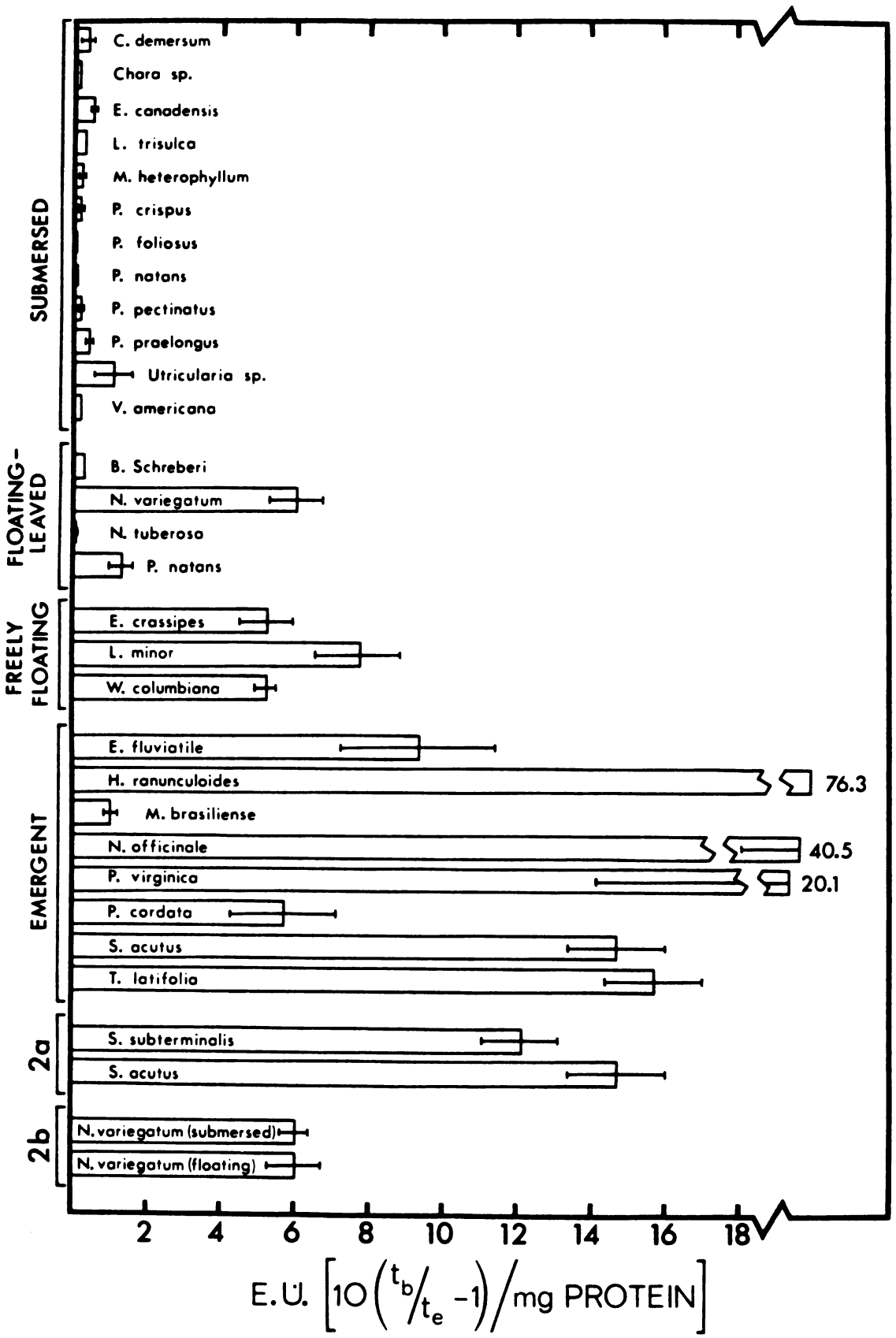
No.	PLANT SPECIES	Carbonic anhydrase activity: E.U. (mg^{-1} protein)	Carbonic anhydrase activity: E.U. (g^{-1} dry weight)	Internal lacunar CO_2 concentration: µl CO_2 g^{-1} dry weight
	Submersed			
1	Ceratophyllum demersum	0.315 ± 0.1596 (3)	44.7 ± 23.52 (3)	545.3 ± 40.12 (4)
2	Chara sp.	0.040 (1)	2.4 (1)	56.8 ± 10.22 (4)
3	Elodea canadensis	0.433 ± 0.0234 (3)	136.2 ± 7.39 (3)	767.4 ± 31.5 (3)
4	Lemna trisulca	0.238 (1)	26.4 (1)	409.0 ± 20.5 (4)
5	Myriophyllum heterophyllum	0.146 ± 0.0690 (3)	58.3 ± 25.46 (3)	297.0 ± 44.36 (4)
6	Nuphar variegatum	6.018 ± 0.3875 (3)	5112 ± 808.0 (3)	241.0 ± 32.20 (4)
7	Potamogeton crispus	0.112 ± 0.0162 (3)	41.2 ± 5.96 (3)	260.0 ± 23.48 (3)
8	Potamogeton foliosus	0 (1)	0 (1)	392.9 ± 21.68 (4)
9	Potamogeton natans	0 (2)	0 (2)	205.8 ± 7.66 (4)
10	Potamogeton pectinatus	0.152 ± 0.0314 (3)	42.6 ± 6.76 (3)	180.8 ± 16.87 (4)
11	Potamogeton praelongus	0.305 ± 0.0642 (3)	132.2 ± 28.31 (3)	301.0 ± 19.24 (4)
12	Scirpus subterminalis	12.102 ± 1.1579 (4)	1654 ± 197.6 (4)	284.4 ± 7.82 (3)
13	Utricularia sp.	1.008 ± 0.5158 (3)	88.9 ± 36.45 (3)	----------
14	Vallisneria americana	0.174 (2)	25.9 (2)	1003.3 ± 125.70 (4)
	Floating-leaved			
15	Brasenia Schreberi	0.240 (1)	204 (1)	----------
16	Nuphar variegatum	6.051 ± 0.7297 (3)	2173 ± 830.6 (3)	58.3 ± 5.00 (3)
17	Nymphaea tuberosa	0.044 ± 0.0246 (3)	64.8 ± 15.33 (3)	74.1 (2)
18	Potamogeton natans	1.285 ± 0.2927 (3)	424 ± 133.1 (3)	229.0 ± 39.00 (4)
	Free-floating			
19	Eichhornia crassipes	5.213 ± 0.7128 (3)	-------	134.6 ± 23.20 (4)
20	Lemna minor	7.724 ± 1.1300 (3)	3073. ± 496.2 (3)	802.9 ± 29.04 (4)
21	Wolffia columbiana	5.216 ± 0.2767 (3)	1446 ± 59.6 (3)	990.4 ± 135.20 (3)
	Emergent			
22	Equisetum fluviatile	9.32 ± 2.077 (3)	2199 ± 488.2 (3)	86.2 ± 2.14 (3)
23	Hydrocotyle ranunculoides	76.44 ± 10.146 (5)	14,762 ± 2324 (5)	137.2 ± 50.75 (4)
24	Myriophyllum brasiliense	1.02 ± 0.126 (5)	698 ± 86 (5)	56.4 ± 5.51 (4)
25	Nasturtium officinale	40.44 ± 1.632 (5)	11,947 ± 476 (5)	----------
26	Peltandra virginica	20.58 ± 5.842 (3)	9,950 ± 1687.2 (3)	96.2 ± 2.63 (3)
27	Pontederia cordata	5.70 ± 1.364 (3)	734 ± 182.4 (3)	79.7 (2)
28	Scirpus acutus	14.72 ± 1.293 (5)	1644 ± 202.7 (5)	81.8 ± 3.96 (3)
29	Typha latifolia	15.86 ± 5.55 (3)	2296 ± 506.6 (3)	155.3 ± 3.60 (3)

FIGURE 2

Carbonic anhydrase activities of aquatic macrophytes across the habitat gradient moving from submersed to floating-leaved and free-floating to emergent plants (E.U. ± SE) (Please refer to Table 1 for entire plant species names.)

2a) Comparison of carbonic anhydrase activities between Scirpus subterminalis, a submersed plant, and Scirpus acutus, an emergent plant.

2b) Comparison of carbonic anhydrase activities between the submersed and floating-leaves of Nuphar variegatum.


SUBMERSED
C. demersum
Chara sp.
E. canadensis
L. trisulca
M. heterophyllum
P. crispus
P. foliosus
P. natans
P. pectinatus
P. praelongus
Utricularia sp.
V. americana
FLOATING-LEAVED
B. Schreberi
N. variegatum
N. tuberosa
P. natans
FREELY FLOATING
E. crassipes
L. minor
W. columbiana
EMERGENT
E. fluviatile
H. ranunculoides
76.3
M. brasiliense
N. officinale
40.5
P. virginica
20.1
P. cordata
S. acutus
T. latifolia
2a
S. subterminalis
S. acutus
2b
N. variegatum (submersed)
N. variegatum (floating)
2
4
6
8
10
12
14
16
18
E.U. [10(tb/te -1)/mg PROTEIN]

the plants in the plant kingdom, i.e., whether the plants were lower plants, monocotyledons, or dicotyledons.

Examination of the carbonic anhydrase activity values within the submersed plant group does not reveal any specific trends with respect to genera. For example, Potamogeton species have carbonic anhydrase values scattered across the whole range of values exhibited by the submersed plant group at the time of sampling. Also, there was no significant difference between the carbonic anhydrase activities of the submersed plants which are able to use HCO_3^- as an inorganic carbon source and Utricularia which is presumably able to use only CO_2 and cannot use HCO_3^-. Hence, the ability of these submersed plants to use HCO_3^- does not appear to be dependent upon the presence of carbonic anhydrase to facilitate the conversion of HCO_3^- to CO_2 prior to photosynthetic fixation. In fact, all of the submersed plants which were assayed that are able to use HCO_3^- had lower carbonic anhydrase activities than Utricularia. Since only one plant known not to use HCO_3^- was assayed and its carbonic anhydrase activity was not significantly higher than the other submersed plants, it is difficult to draw any generalizations about the function of carbonic anhydrase in these two groups of plants.

Carbonic anhydrase activities within the floating-leaved aquatic plant group were variable. Nuphar variegatum had a rather high carbonic anhydrase activity while the floating-leaves of Potamogeton natans had a lower

level, but was still higher than any found among the submersed plants. Nymphaea tuberosa and Brasenia Schreberi, however, had very low carbonic anhydrase activities--as low as plants within the submersed plant group. It is possible that these carbonic anhydrase activities are related to the productivities of these plants, as is discussed below.

The carbonic anhydrase activities of emergent plants were quite consistently high with the exception of the low activity found in Myriophyllum brasiliense. Some of the variation among the emergent plant species perhaps can be related to differences in productivity (see below).

Some evolutionary relationships appear to exist between the heterophyllous leaves of certain aquatic plant species and between species which grow in different habitats but belong to the same genera. The carbonic anhydrase level of the submersed leaves of Nuphar variegatum was six times higher than the carbonic anhydrase levels found in the leaves of the other submersed plants examined, but was equivalent to the activity detected in the floating-leaves of the same plant (Figure 2, part b). Similarly, although the carbonic anhydrase level detected in the floating-leaves of Potamogeton natans was higher than that detected in the submersed leaves of this same plant, the levels observed in the floating-leaves were still much lower than those found in the floating-leaves of N. variegatum (Figure 2). The carbonic anhydrase levels found in the submersed Scirpus

subterminalis were twelve times higher than the levels observed in the other submersed aquatic plants examined, but were nearly as high as the activities detected in the emergent Scirpus acutus (Figure 2, part a). Myriophyllum brasiliense, an emergent aquatic macrophyte, expressed a carbonic anhydrase activity that was much lower than that observed in the other emergent macrophytes examined. Its carbonic anhydrase activity was nearly as low as the submersed Myriophyllum heterophyllum (Figure 2). These examples may provide evidence for an evolutionary biochemical relationship between these plants which extends beyond the more obvious morphological and anatomical characteristics. Hence, even though the submersed leaves of N. variegatum and S. subterminalis more closely resemble the submersed leaves of the other submersed plants, the carbonic anhydrase activities of these leaves suggests a biochemical relationship of the submersed leaves to the floating-leaves of N. variegatum and of the submersed S. subterminalis to the emergent S. acutus of the same genus. Similarly, even though the floating-leaves of P. natans and the emergent portion of M. brasiliense resemble other floating-leaves and emergent plants in their respective morphological and anatomical characteristics, the floating-leaves of P. natans actually display a closer biochemical relationship on the basis of carbonic anhydrase activity to the submersed leaves of the same plant species, and

M. brasiliense displays a closer relationship to M. heterophyllum of the same genus.

The general trend in carbonic anhydrase activity over the habitat gradient appears to be related to the productivities of these plants. Submersed plants, which were low in carbonic anhydrase activity, have low net production rates in comparison to emergent plants, which have much higher carbonic anhydrase activities and high rates of production. (For production rates see Wetzel, 1975 and Westlake, 1963.) The relationship between seasonal maximum biomass and carbonic anhydrase activity is plotted in Figure 3. In speaking of the productivities of these plants, seasonal maximum biomass may be used as a relative measure of plant productivity. The low carbonic anhydrase activities observed in submersed plants could account partly for the low productivities of these plants. One cannot, however, necessarily draw the conclusion that the low productivities of submersed plants are solely because of low carbonic anhydrase levels. Other factors and processes operate to reduce the net production rates in submersed plants in comparison to emergent plants. For example, the light limitations which are inherent to a submersed existence reduce photosynthetic rates and the excretion of organic compounds from these plants incurs a great loss to the gross production rate (Wetzel, 1975).

A relationship between carbonic anhydrase levels and productivity of aquatic plants could explain several of the

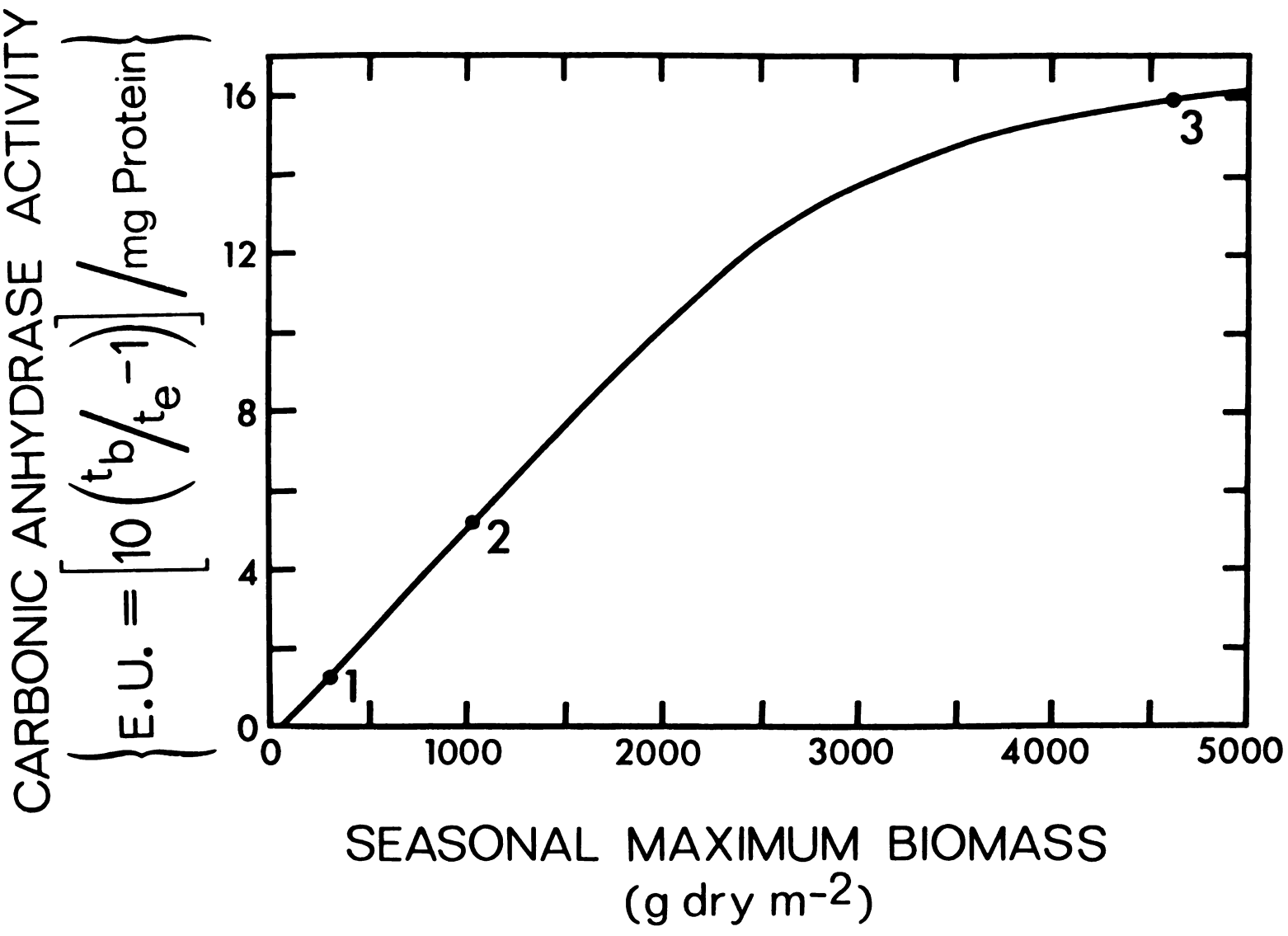
FIGURE 3

Carbonic anhydrase activity versus seasonal maximum biomass for aquatic macrophytes

1. Carbonic anhydrase value is $\bar{x}$ of all submersed plants examined. A seasonal maximum biomass of 300 g dry m^{-2} was used. This value is intermediate the values reported by Rickett, 1921; and Rich, Wetzel, and Thuy, 1971; for submersed aquatic plants in hardwater lakes.

2. Carbonic anhydrase value is $\bar{x}$ of n=3 for Eichhornia crassipes, a free-floating plant. A seasonal maximum biomass of 1000 g dry m^{-2} was used. This value is midway those values reported by Penfound and Earle, 1948.

3. Carbonic anhydrase value is $\bar{x}$ of n=3 for Typha latifolia. A seasonal maximum biomass of 4640 g dry m^{-2} was used (Bray et al., 1959).


CARBONIC ANHYDRASE ACTIVITY
{E.U. = [10 (tb/te -1)] /mg Protein}
0
4
8
12
16
0
1000
2000
3000
4000
5000
1
2
3
SEASONAL MAXIMUM BIOMASS
(g dry m-2)

inconsistencies observed in the carbonic anhydrase data. As noted earlier, Nymphaea tuberosa and Brasenia Schreberi, both floating-leaved plants, exhibited much lower carbonic anhydrase levels than the leaves of Nuphar variegatum. Nuphar is nearly always much more productive and possesses a much larger biomass than either Nymphaea or Brasenia in nature. N. tuberosa plant densities were much lower in Lawrence Lake than were N. variegatum densities, indicating much higher biomass levels of N. variegatum than for N. tuberosa. In Duck Lake, B. Schreberi also exhibited much lower plant densities than N. variegatum. In both cases, N. variegatum was very definitely the dominant plant in the floating-leaved zone and was extremely productive. Perhaps the low levels of carbonic anhydrase in N. tuberosa and B. Schreberi could be a factor in the low productivity of these plants.

A similar observation of the relative plant densities of Pontederia cordata and Peltandra virginica may explain the carbonic anhydrase data for these two species. Both of these plants were collected from Three Lakes. P. virginica demonstrated significantly higher plant densities and biomass than did P. cordata in this lake. The carbonic anhydrase levels of P. cordata, which were four times less than those exhibited by P. virginica, may again partly account for the lower productivity level observed in P. cordata.

The mean values for replicates of each plant species for both carbonic anhydrase activity and internal CO_2

concentrations are given in Figure 4 for each habitat group (submersed, floating-leaved, free-floating, and emergent). No specific trend in carbonic anhydrase activity versus internal CO_2 concentration for plants across the habitat gradient emerged. In general, submersed plants all had low carbonic anhydrase activities, but exhibited a range of internal CO_2 concentrations from low to high. Emergent plant species showed relatively high carbonic anhydrase activities, but low internal CO_2 concentrations. Floating-leaved and leaves of free-floating plants had intermediate carbonic anhydrase levels. Internal CO_2 concentrations of floating-leaves tended to be low and within the same range as that shown by emergent plants, but internal CO_2 concentrations of free-floating plants varied over a wider range.

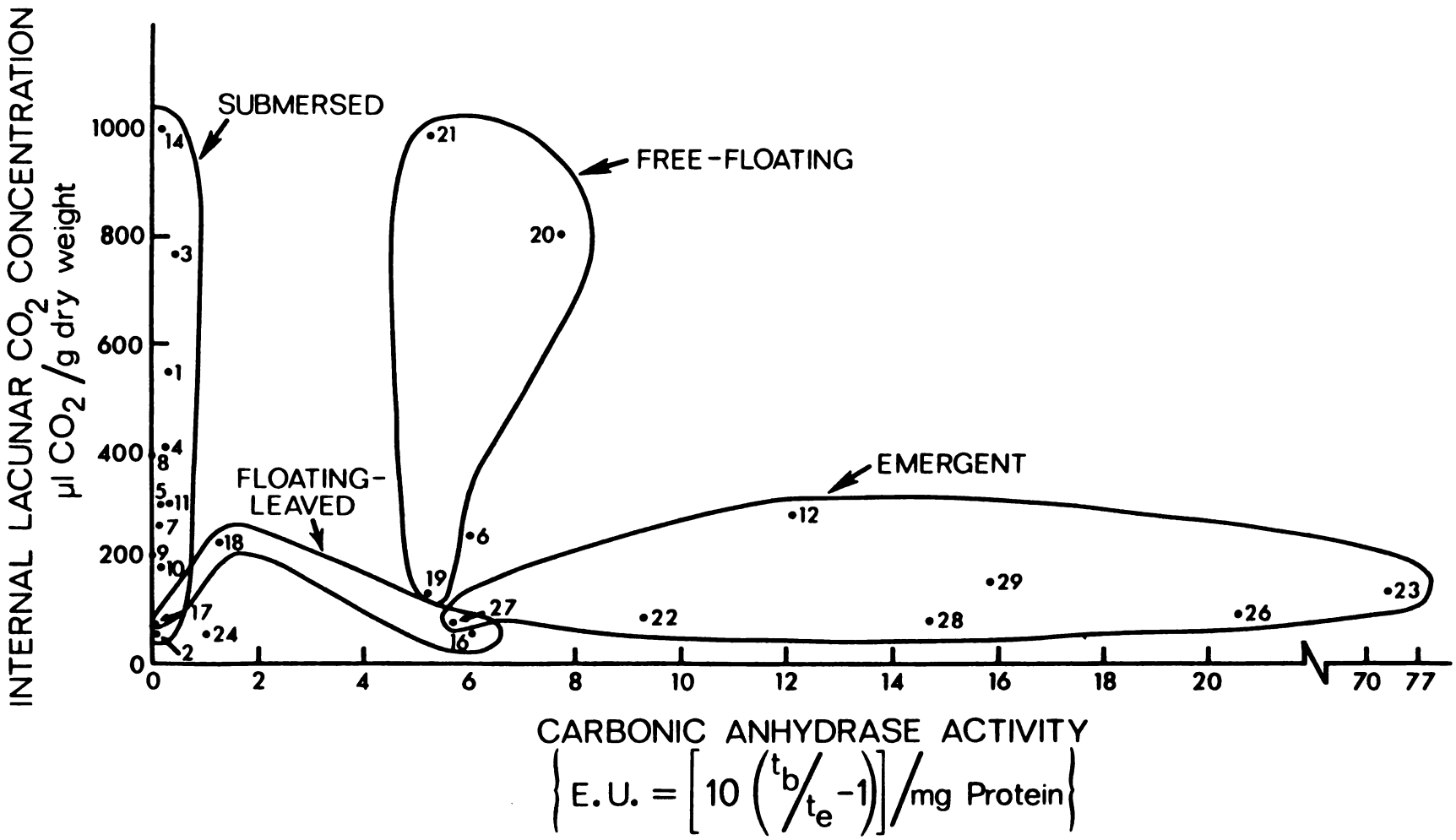
Both external environment and morphological and anatomical characteristics of these plants may explain some of the differences observed between the internal CO_2 concentrations of different aquatic plants. These morphological and anatomical characteristics can be examined with regard to leaf shape and thickness and the presence or absence of stems and rhizome structures.

With regard to the effect of external environment on internal CO_2 concentrations, it may be difficult to draw a clear picture of the dynamics of gas exchange between the internal lacunar gas system and the external environment because of the special morphological and anatomical

FIGURE 4

Carbonic anhydrase activity versus internal lacunar CO_2 concentrations for aquatic macrophytes

Submersed, floating-leaved, free-floating, and emergent aquatic macrophyte groups are shown within envelopes. The numbers correspond to the species listed in Table 4.


INTERNAL LACUNAR CO_2 CONCENTRATION
µl CO_2 /g dry weight
SUBMERSED
FREE-FLOATING
FLOATING-LEAVED
EMERGENT
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
70
77
CARBONIC ANHYDRASE ACTIVITY
$\left\{ E.U. = \left[10 \left({}^{t_b}/_{t_e} - 1 \right) \right] / \text{mg Protein} \right\}$

adaptations to their environment which plants from each of the habitats possess. For example, leaves of submersed plants are very thin (only 1-3 cells thick) and either long and linear or very finely dissected (Sculthorpe, 1967; Wetzel, 1975). In addition, submersed leaves lack a cuticle. These adaptive characteristics act to facilitate gas transport between the internal lacunar system and the water. Since diffusion readily occurs across the leaf surface, stomata are not required and only vestiges of a stomatal apparatus are observed in the leaves of these plants. In contrast, emergent plants have thicker, cuticularized leaves with stomata. In these plants gas diffusion must occur through the stomata. The external environment, however, greatly affects the diffusion of gases. Submersed plants must exchange gases with an aqueous medium which greatly slows gas diffusion, and a boundary layer of unstirred water lies immediately adjacent to the leaf surface of submersed plant leaves making gas diffusion into and out of the leaves even more difficult (Wetzel, 1975). In contrast, though emergent plants appear to have more morphological and anatomical impediments to gas diffusion, gas diffusion actually may be easier since gases diffuse directly into a gaseous atmosphere. In addition, emergent plants can "afford" to keep their stomata open constantly as they do not have to be concerned with excessive water loss. Therefore, gas diffusion into and out of the lacunar system of emergent plants is much more rapid than for submersed plants

(Søndergaard and Wetzel, 1979). Hence, it would be reasonable to assume that the internal CO_2 concentrations of emergent plants would be lower than those of submersed plants. This relationship also was illustrated by the internal CO_2 concentrations of emergent and submersed plants (Table 4). All of the submersed plants, with the exception of Chara which lacks an internal lacunar system, had higher internal CO_2 concentrations than the emergent plants.

The internal gas composition of leaves also may be affected by rhizomes. These structures are buried in anaerobic mud and demonstrate a high O_2 respiratory demand. This O_2 is most likely supplied by photosynthesis in the foliage which diffuses down to the underground organs (Wetzel, 1975). In return, CO_2 produced by respiration in these underground organs diffuses upward into the aerial portions of the plant (Wetzel and Penhale, 1979). Because of this potentially large source of CO_2, the leaves of aquatic plants that possess underground organs tend to have higher internal CO_2 concentrations than would those aquatic plants lacking these underground structures.

The submersed plant group exhibited a wide range of internal CO_2 concentrations. Within the submersed plant group, Chara had the lowest internal CO_2 concentration, as would be expected since the macroalga Chara lacks an internal lacunar system and does not accumulate CO_2 internally.

Among the Potamogeton spp., internal CO_2 concentrations increased from P. pectinatus to P. natans to P. crispus to P. praelongus to P. foliosus. This trend may be explained partly as follows. P. pectinatus has long linear, very thin leaves. This combination of leaf characteristics would constitute a high surface area to volume ratio and hence probably allow moderately rapid diffusion of gases, including CO_2, out of the leaves. P. natans also has long and narrow leaves, but they are thicker which may slow CO_2 diffusion out of leaves and hence explain the slightly higher internal CO_2 levels which were observed in this plant species in comparison to P. pectinatus. P. crispus and P. praelongus both have flat broad leaves. This broader form could result in lowered CO_2 diffusion out of the leaves (a lower surface area to volume ratio than in P. pectinatus and P. natans) and hence greater internal CO_2 concentrations. The finely toothed margin of P. crispus may give a slightly greater surface area to volume ratio than would be present for P. praelongus and may explain the slightly lower internal CO_2 concentrations that were observed in P. crispus. P. foliosus exhibited the highest internal CO_2 concentration. Its leaf form is thin and narrow and so it would have been expected to have a lower internal CO_2 concentration. The leaves of P. foliosus, however, had a thicker encrustation of calcium carbonate on its leaves than any of the other submersed plants. This encrustation could have decreased gas diffusion out of the leaves and therefore resulted in

higher internal CO_2 concentrations within the leaves. In this way, calcium carbonate precipitated on leaf surfaces could be very detrimental in terms of gas exchange for submersed aquatic plants which are able to use bicarbonate for photosynthesis.

Myriophyllum heterophyllum has highly dissected leaves and exhibited moderate internal CO_2 levels. CO_2 diffusion from these leaves is probably relatively good because of the high surface area to volume ratio. Ceratophyllum demersum also has highly dissected leaves, but showed higher internal CO_2 concentrations than did M. heterophyllum. The leaf segments of C. demersum are thicker than the leaf segments of M. heterophyllum, which may have resulted in slower diffusion of gases out of the lacunar system of C. demersum and may account for the higher observed internal CO_2 levels. In addition, the growth form of these two plant species may explain part of the difference in internal CO_2 levels. C. demersum tends to grow in dense mats and to lie along the sediments, which may be anaerobic. In contrast, M. heterophyllum tends to grow vertically up through the water column. The fact that C. demersum tends to grow along the sediments may mean there is less diffusion of gases out of the lacunae so that gases could have accumulated within the lacunae. CO_2 may have especially accumulated because of the plants' proximity to the sediments (CO_2 evolved by the decomposition of organic matter) and

because of enhanced respiration from light limitations and density of the plant material.

Elodea canadensis had higher internal CO_2 levels than either M. heterophyllum or C. demersum. The leaves of E. canadensis are broader than those of either M. heterophyllum or C. demersum, but are very small. Hence, leaf shape would not seem to explain the high internal CO_2 levels observed in E. canadensis. Sediment conditions in which this plant was rooted could perhaps partially explain these high internal CO_2 levels. The sediments appeared to be very high in organic matter and the plants were not growing very high above the sediments. Thus, the proximity of these plants to a high source of dissolved CO_2 from the decomposition of organic material may have led to increased intrinsic CO_2 levels within the plant.

Vallisneria americana exhibited the highest internal CO_2 concentration of all of the submersed plants. The leaves of V. americana are long and ribbon-like and grow as a rosette from an underground stem. The growth of this stem underground could mean that CO_2 may have accumulated within the stem and directly diffused up into the leaves, resulting in higher internal CO_2 concentrations than were observed in the other submersed plants. Scirpus subterminalis has a growth form similar to V. americana, but exhibited three times less internal CO_2 than was observed in V. americana. The lower CO_2 level observed in S. subterminalis may be because its leaves are much longer and thinner than the

leaves of V. americana and because it also has a lesser developed internal lacunar system. Its thinner leaves would constitute a higher surface area to volume ratio and hence would allow better diffusion of gases out of the plant and result in less internal accumulation of CO_2 than occurs in V. americana.

Of the floating-leaved species examined, Nuphar variegatum and Nymphaea tuberosa had quite low internal CO_2 concentrations whereas the floating-leaves of Potamogeton natans exhibited higher levels. N. tuberosa exhibited slightly higher internal CO_2 levels than did N. variegatum. Field observation of these two species revealed that the rhizome of N. tuberosa was typically rooted deeper in the sediments than was the rhizome of N. variegatum. This growth form could perhaps have resulted in a greater accumulation of respired CO_2 within the rhizome so that more CO_2 was observed within the leaves of N. tuberosa as this CO_2 diffused upward. P. natans exhibited even higher levels, perhaps resulting from the transport of CO_2 from the submersed foliage of the plant into the floating leaves.

Of the three free-floating plants examined, Lemna minor and Wolffia columbiana had very high levels of internal CO_2 whereas Eichhornia crassipes had much lower levels. E. crassipes has inflated petioles which project several inches above the water surface. Both L. minor and W. columbiana, however, have very small inflated leaves which lie directly on top of the water. Hence, the rate of

CO_2 diffusion out of the leaves of L. minor and W. columbiana is probably slowed greatly so that CO_2 levels would build up within the plant. In the case of E. crassipes, however, the projection of the inflated petioles and leaves above the water would probably have allowed for greater diffusion of gases out of the plant and resulted in lower CO_2 concentrations within the leaves.

The internal CO_2 concentrations of leaves of the emergent plants were all fairly low. Myriophyllum brasiliense showed the lowest internal CO_2 concentration of the emergent plants. Unlike most of the other emergent plants, it lacks an underground rhizome and hence would not have experienced foliar accumulation of CO_2 that had diffused upward as a result of respiration in the rhizome. In addition, gas diffusion out of the finely dissected leaves of M. brasiliense is probably very rapid so that little CO_2 accumulation occurs. Of the other emergent plants, Scirpus acutus possesses a smaller and more shallowly rooted rhizome than Typha latifolia. The lower internal CO_2 concentration exhibited by S. acutus in comparison to T. latifolia could have been a reflection of this.

The above discussion points out: (1) the differences between the levels of carbonic anhydrase in aquatic macrophytes, (2) considers the effect of internal lacunar CO_2 concentrations on carbonic anhydrase levels in these plants, and (3) examines the relative internal CO_2 concentration of these plants with regard to their respective morphologies

and environments. The results of this investigation, however, still can not address the question of what the actual function of carbonic anhydrase is in plants. The following discussion of the possibilities of its function in plants with special emphasis on submersed aquatic plants may provide some insight into this question.

It has been suggested that carbonic anhydrase may function as a permease to facilitate the diffusion of CO_2 into plants (Graham and Reed, 1971). If this is so, it would appear that the presence of the enzyme in submersed aquatic plants would be of immense importance in facilitating the diffusion of CO_2 dissolved in water into the leaves. Such an enhancement of CO_2 diffusion would especially seem to be of significance in view of the slow rates of CO_2 diffusion in water. Hence, the fact that carbonic anhydrase levels in submersed aquatic macrophytes are low may in itself provide evidence against the proposed permease function of carbonic anhydrase in plants. But, perhaps the typical perception of the diffusive limitations of CO_2 into submersed plants should be examined in a different way. Since the leaves of submersed plants are very thin, lack a cuticle, and possess a chloroplastic epidermis, the limitations to CO_2 transport into the plant cells may not actually be very great. The limitations to CO_2 transport for submersed aquatic macrophytes, therefore, are probably not a problem of actual diffusion of CO_2 into the plant cells, but more likely, of the diffusive limitations of CO_2

getting to the leaf surface. Since the role of carbonic anhydrase as a permease would involve the former (the diffusion of CO_2 into the plant) rather than the latter, (the diffusion of CO_2 to the plant), then it is reasonable to expect that any action of carbonic anhydrase as a permease in these plants would be of no great value.

A possible function of carbonic anhydrase is in the re-fixation of respired and photorespired CO_2 in C_3 plants. As was discussed by Everson and Slack (1968), carbonic anhydrase has been visualized as perhaps functioning to scavenge respired and photorespired CO_2 in C_3 plants in the same kind of way that PEPcase scavenges and facilitates the re-fixation of CO_2 in C_4 plants. The lacunar spaces of submersed aquatic macrophytes accumulate respired and photorespired CO_2 and may serve in this same function. This function of the internal lacunar system appears to be one of utmost value to these plants as net photosynthetic fixation of CO_2 in the submersed Scirpus subterminalis declines sharply in the absence of this internal lacunar system (Søndergaard and Wetzel, 1979). Hence, this lacunar system could very well retain respired and photorespired CO_2 long enough to facilitate the re-fixation of this CO_2 and eliminate the need for special enzymes, such as carbonic anhydrase or PEPcase, to carry out this function. This would accentuate the possible importance of the lacunar system to the re-fixation of CO_2 and consequently, the possible unimportance of carbonic anhydrase in a similar role.

Carbonic anhydrase also has been postulated to function in ion transport and some evidence has been presented in support of this theory (Findenegg, 1974, Rybová and Slavíková, 1973). In these studies, carbonic anhydrase was associated with the transport of HCO_3^-, Cl^-, K^+, and Na^+ in algae. A review of ion transport in submersed aquatic plants may provide some insight into possible reasons for the low carbonic anhydrase activities observed in these plants in comparison to the higher activities observed in algae. The leaves of submersed aquatic macrophytes, like the cells of algae, are able to take up ions from the water (Wetzel, 1975). The mechanisms differ, however: submersed aquatic macrophytes and the leaf underside of floating-leaved plants, unlike algae, possess special "organs" called hydropoten which function to actively take up ions. If carbonic anhydrase does function in ion transport, the ability of submersed leaves to take up ions by hydropoten may suppress synthesis of carbonic anhydrase and therefore could explain the extremely low levels observed in these plants in comparison to algae. This reasoning also could explain the intermediate carbonic anhydrase levels observed in floating species as these species would not have the same benefits of being able to take up ions through both leaf surfaces. It is difficult, however, to relate these concepts to how carbonic anhydrase might function in emergent and terrestrial plants. Perhaps the fact that submersed aquatic macrophytes can obtain ions from both the roots and

the leaves whereas emergent and terrestrial plants are able only to obtain ions through the roots, means that submersed plants are able to maintain an ionic balance throughout the plant structure much easier than emergent or terrestrial plants can. Hence, what could become an "ionic stress" in emergent and terrestrial plants may be prevented by carbonic anhydrase operating in an ion transport capacity.

In summary, carbonic anhydrase levels vary among aquatic macrophytes, and in general, are low in submersed plants and higher in emergent plants. Floating-leaved and free-floating plants demonstrate intermediate carbonic anhydrase levels. The apparent trend is that carbonic anhydrase levels increase moving across the habitat gradient from submersed to emergent plants. Some exceptions to this general trend were evident.

Internal CO_2 concentrations of these plants were not related to carbonic anhydrase activities as had been postulated at the onset of this investigation. Internal CO_2 concentrations of submersed plants ranged widely whereas the internal CO_2 concentrations of all the emergent plants were low in comparison. Further, the differences in internal CO_2 concentration data within groups appeared to be related to the morphologies of the individual plant species and, also to an extent, the environments in which they grew.

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