Plant Physiol. (1975) 55, 468-474

Carbonic Anhydrase of Spinach

STUDIES ON ITS LOCATION, INHIBITION, AND PHYSIOLOGICAL FUNCTION[1]

Received for publication August 1, 1974 and in revised form October 4, 1974

BRUCE S. JACOBSON,[2] FRANKLIN FONG, AND ROBERT L. HEATH
Departments of Biology and Biochemistry, University of California, Riverside, California 92502

ABSTRACT

Carbonic anhydrase activity was determined in spinach (*Spinacia oleracea*) leaf organelles isolated on sucrose density gradients and was found to be predominantly in the intact chloroplast fraction. The small amount of activity associated with the mitochondrial fractions was probably due to intact chloroplast contamination. No activity could be associated with the broken chloroplast or microbody fractions. Based upon inhibitor studies, carbonic anhydrase was found to be around 2 mM in the chloroplast. Ethoxzolamide, an inhibitor of carbonic anhydrase, reduced CO_2 fixation in intact chloroplasts. The concentration required to inhibit CO_2 fixation 20 to 40% was in excess of that required to inhibit the purified enzyme. The inhibition was partially reversed by CO_2. Ethoxzolamide had no effect on photosynthetic NADP reduction or photophosphorylation measured by methyl viologen reduction. The physiological role of carbonic anhydrase was shown not to be associated with CO_2 diffusion or CO_2 concentration. It is proposed that other functions of carbonic anhydrase could be the protection against denaturation by transient localized changes in pH or the hydration of compounds other than CO_2.

Although carbonic anhydrase is well known to be present in green plants, its role in efficient photosynthesis has been seriously discussed only recently (8, 10). As previously pointed out, bicarbonate ion, because of its solubility, would make an effective reservoir of CO_2 for photosynthetic carboxylation (31). However, bicarbonate ion could function as a counterion for transport coupled with protonic and cationic movements (8). The rapid intraconversion of CO_2 into HCO_3^- would require the presence of carbonic anhydrase. Thus, the cellular localization and metabolic role of this enzyme has considerable interest as to bicarbonate/CO_2 movements.

With nonaqueous (9) and differential centrifugation techniques (21), carbonic anhydrase activity has been found to be associated with the intact chloroplasts and the cytosol of higher plants. Unfortunately, the centrifugation velocities previously used in localizing carbonic anhydrase in plants were high enough to pellet significant amounts of mitochondria as well as intact chloroplasts (21). Whether other plant organelles contain carbonic anhydrase, and where in the chloroplast is carbonic anhydrase located, is not completely solved and, thus, is part of this study.

In addition, the finding that acetazolamide inhibited photosynthetic electron transport as well as carbonic anhydrase seriously questions whether or not the acetazolamide inhibition of photosynthetic CO_2 fixation was a function of carbon anhydrase activity. This paper is further concerned with the study of another inhibitor or carbonic anhydrase—ethoxzolamide—with respect to its inhibitory kinetics and its effect upon isolated intact chloroplasts.

MATERIALS AND METHODS

The leaves of washed, commercial spinach (*Spinacia oleracea*), after deribbing, were chopped gently with razor blades in a grinding medium of 400 mM sorbitol, 20 mM HEPES buffer (pH 8.0), 10 mM $MgNa_2$-EDTA, 1 mM dithiothreitol, and 1% bovine serum albumin at 0 C (1.5 ml of grinding medium per g fresh weight). The leaf homogenate was then gently ground in a mortar and pestle at 0 C which contained about 0.5 ml of partially frozen grinding medium per g fresh weight of leaves.

The slurry was then filtered once through a 30-μm diameter nylon mesh to remove cell wall debris and unbroken cells. The supernatant of this solution obtained from a low speed centrifugation (either 1,000g or 3,000g) was then layered onto sucrose density gradients, as previously described (24), with the following modifications. Pellets were resuspended in grinding medium without $MgNa_2$-EDTA, and 1 mM dithiothreitol was substituted for mercaptoethanol. The gradients were centrifuged at 25,000 rpm in a Beckman SW27 swinging bucket rotor in a Beckman L2-50 ultracentrifuge.

Isolated intact spinach chloroplasts, used for the inhibitor studies, were prepared as previously described (12). Published methods were used to assay for glyceraldehyde-3-P dehydrogenase, malate dehydrogenase (24), and chlorophyll concentrations (1). The carbonic anhydrase assay consisted of a reaction mixture of 4.0 ml of barbituric acid buffer (22.5 mM at pH 9.0), 0.1 ml of enzyme suspension, and 0.9 ml of distilled water. Blanks were either 0.1 ml of boiled enzyme suspension or distilled water. The reaction was followed by recording the pH changes on a Radiometer pH meter (type TTT1c) and a Bausch and Lomb recorder. The dehydration activity ($HCO_3^- + H^+ \rightarrow H_2O + CO_2$) was calculated from the time required to change the pH of the buffer from 7.5 to 6.8, after subtracting the time required to change the pH of the blank (no enzymes).

The carbonic anhydrase activity was calculated for the sucrose gradients using a pH change from 7.3 to 6.0.

The CO_2 concentration in solution was calculated according to the Bunsen coefficients (29). Typical experiments in-

[1] This research was supported in part by federal funds of the United States Environmental Protection Agency (EPA R-801311).

[2] Present address: The Biological Laboratories, 16 Divinity Avenue, Harvard University, Cambridge, Mass. 02138.

volved adding aliquots of water bubbled with 100% CO_2 to the various solutions.

All photosynthetic measurements were made by following the production of O_2 with an oxygen electrode. The reaction vessel containing 3 ml of the chloroplast suspensions was kept at 25 C and 3 cm away from a 500-w tungsten filament lamp. All rates were determined during a 1- to 3-min interval following a 2- to 5-min equilibration period. The reaction mixture used for the determination of either PGA[3] or CO_2-dependent photosynthetic O_2 evolution contained 300 mM sorbitol, 50 mM Tricine at pH 7.6, 1 mM Pi, 1 mM Na-isoascorbate, and 75 μg Chl ml^{-1}. The effect of ethoxzolamide on photophosphorylation was estimated by determining the change in O_2 concentration of chloroplast suspensions in the presence and absence of 2 mM NH_4Cl. The reaction medium contained 0.1 m methyl viologen, 1.5 mM ADP, 1.5 mM Pi, 10 mM KCl, 1 mM KCN, 50 mM Tricine at pH 7.6, and chloroplasts equivalent to 50 μg of Chl/ml of medium.

Photosynthetic NADP reduction was measured as previously described (27). The reaction medium contained 30 mM Tricine at pH 7.6, 1 mM $MgCl_2$, 1.5 mM NADP, 45 μg ml^{-1} ferredoxin, 40 μg ml^{-1} NADP-ferredoxin reductase, and 20 μg ml^{-1} Chl. NADPH produced during three successive 10-min intervals was determined spectrophotometrically.

RESULTS

Distribution of Carbonic Anhydrase in Subcellular Fractions. When spinach leaf organelles were separated on a linear sucrose gradient only one major peak of carbonic anhydrase activity could be observed (Fig. 1). Figure 1A is the 1,000*g* pellet applied to a 40 to 80% (w/v) sucrose density gradient, whereas Figure 1B is the 3,000*g* pellet applied to a similar gradient. The 1,000*g* pellet had a greater percentage of intact chloroplasts (24), generally 50 to 70%. Marker enzymes are GPDH for intact chloroplasts, and malic dehydrogenase for mitochondria and microbodies (24). Note that in both figures the carbonic anhydrase activity roughly followed that of the GPDH activity even though the activity of CA in Figure 1B was 20-fold lower. Also, in Figure 1B the fractions corresponding to the mitochondrial band showed only trace amounts of carbonic anhydrase activity. This trace amount is most likely due to intact chloroplast contamination, since the GPDH activity (for which the assay is more sensitive) indicates some intact chloroplasts were present.

Mitochondria did not inhibit carbonic anhydrase activity as tested by adding (*a*) boiled leaf mitochondrial fractions from a sucrose density gradient and (*b*) boiled and unboiled spinach root mitochondria (obtained by centrifuging a whole homogenate of root at 1,000*g* to 15,000*g* for 30 min) to a standard carbonic anhydrase assay.

When intact chloroplasts were lysed by adding an equal volume of grinding medium without sorbitol to intact chloroplasts removed from a 40 to 80% sucrose density gradient, no carbonic anhydrase activity was associated with the chlorophyll peak (Fig. 1C). This confirms previous findings (21) that carbonic anhydrase is not associated with the grana membranes. Furthermore, when the intactness of a chloroplast preparation was taken into account, most of the carbonic anhydrase activity was considered to be associated with the chloroplast (30). Also, fractionation of spinach organelles by nonaqueous isolation procedures indicated that the carbonic anhydrase activity to chlorophyll ratio was essentially a constant (9). When we made corrections for the percentage of intact chloroplasts, the carbonic anhydrase activity per unit chlorophyll of the total leaf homogenate was the same as that for the chloroplasts isolated from the same tissue source. Thus, while it has been reported that only 63% of the spinach leaf carbonic anhydrase was chloroplastic (21), we now confirm earlier suggestions (10, 30) that most, if not all, of the enzyme was within the chloroplast stroma.

The sucrose density gradient method for the isolation of spinach leaf organelles has shown that carbonic anhydrase activity was associated only with the intact chloroplasts and was found neither in the stripped chloroplasts, outer membrane of chloroplasts, mitochondria, nor microbodies.

Carbonic Anhydrase and Its Inhibition by Ethoxzolamide. Carbonic anhydrase activity increased in direct proportion to enzyme concentration over the concentration range that was used in all our experiments. The *Km* for CO_2 hydration established by reciprocal plots of substrate *versus* activity was 29.9 mM—a value similar to that reported for the enzyme from pea (15) and parsley (28).

Since ethoxzolamide is likely bound to carbonic anhydrase with a one to one stoichiometry (17), this inhibitor could be used to inhibit the enzyme in the chloroplasts. Inhibition of spinach carbonic anhydrase by ethoxzolamide appeared quite effective (Fig. 2A). The type of inhibition for ethoxzolamide was found to be competitive (Fig. 2B) with a *Ki* of 0.5 to 0.8 μM at levels of inhibition greater than 40%. At lower inhibitory levels the type of inhibition appeared noncompetitive with a *Ki* of about 0.2 μM.

Lamellar fragments did not alter the degree of inhibition of carbonic anhydrase by ethoxzolamide. Similarly, the enzyme purified by isoelectronic focusing (20-fold; pI = 4.90) was the same as the crude preparation with regard to inhibitor sensitivity. It is, therefore, likely that the binding of ethoxzolamide was specific for carbonic anhydrase. The *Ki* was significantly less than that for the inhibitor-carbonic anhydrase complex of acetazolamide, a compound that also blocked photosystem II (9) and, therefore, must have been bound not only to the anhydrase but to the photosystem as well. Also, the additions of lipid-protein complexes in the chloroplast fragments did not reduce the extent of inhibition as they would have if the inhibitor bound nonspecifically.

Turnover and Concentration of Carbonic Anhydrase in Chloroplast. Since all of the carbonic anhydrase in spinach leaves was in the chloroplast, it became of interest to determine just how much there was. The amount can be determined from kinetic studies of ethoxzolamide inhibition using the method of Strauss and Goldstein (26) or by titrating the enzyme activity with inhibitor. The first procedure is depicted in Figure 3 and the second in Fig. 4. The first method evaluated the data at high levels of inhibition, whereas the second put more weight on the lower levels. While both methods yielded turnover numbers in the range of from 40 to 80 mmoles CO_2 hydrated min^{-1} per μmole of carbonic anhydrase, the titration method gave about one-half the standard error. It is important to note that although these turnover numbers can be used for determination of enzyme concentration, they do not necessarily reflect the true catalytic constant. Furthermore, the inhibitor method is based upon the assumption that the stoichiometry of inhibition by ethoxzolamide is the same in the spinach enzyme as with other carbonic anhydrases (17).

With the above information, the amount of enzyme per unit chlorophyll for intact chloroplasts was found to be from 0.07 to 0.1 μmole/mg for chloroplasts isolated on sucrose

[3] Abbreviations: PGA: phosphoglyceric acid; CA: carbonic anhydrase; "CO_2": all species of CO_2, H_2CO_3 and H^+, HCO^-_3 taken collectively; GDPH: NADP glyceraldehyde-3-phosphate dehydrogenase.

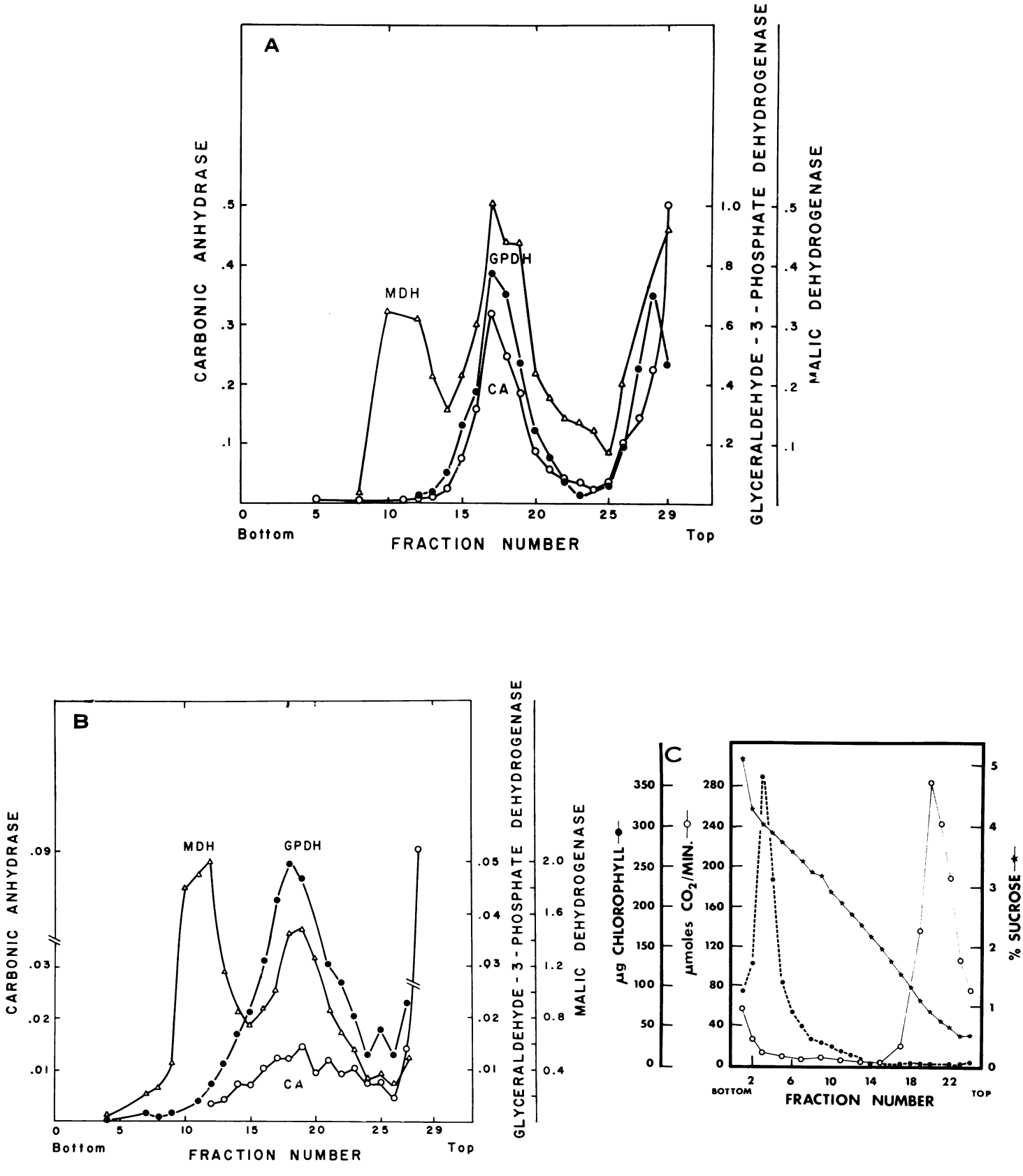

FIG. 1. Separation of organelles from spinach leaves on a sucrose gradient. A: 1000*g* pellet starting material; B: 3000*g* pellet starting material; C: lysed chloroplast starting material. A and B: Carbonic anhydrase (ΔpH sec^{-1}), NADP-glyceraldehyde-3-phosphate dehydrogenase (ΔA_{340} min^{-1}), malic dehydrogenase (ΔA_{340} min^{-1}); C: carbonic anhydrase activity expressed as μmoles CO_2 min^{-1}.

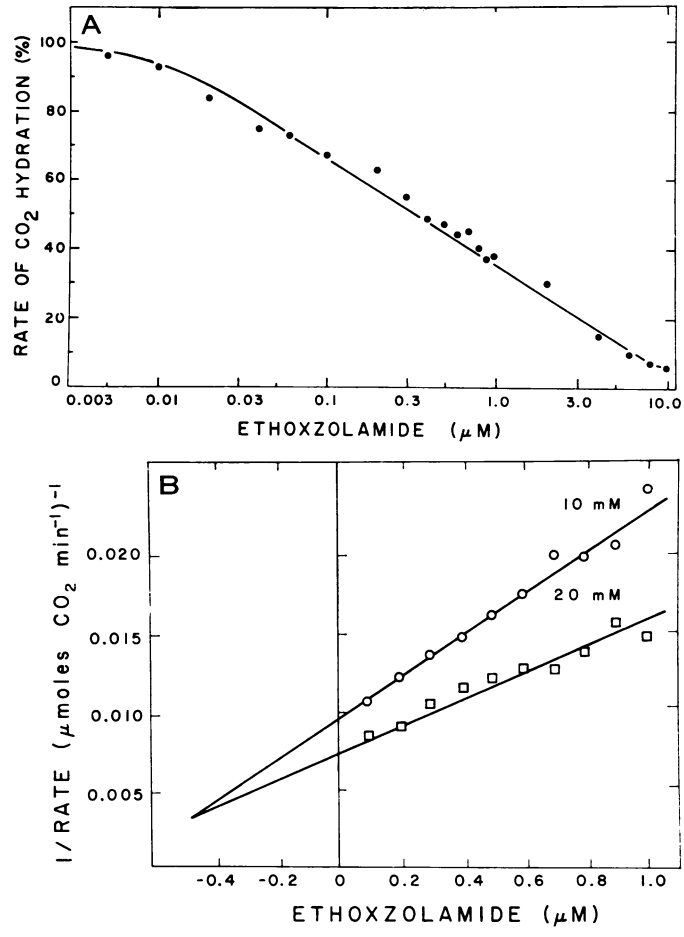

FIG. 2. Inhibition of spinach carbonic anhydrase by ethoxzolamide. A: All assays with 20 mM CO_2; B: CO_2 at 10 and 20 mM as indicated.

gradients depicted in Figure 1A. Since the chloroplast volume is about 30 μl (13), the concentration of carbonic anhydrase in the chloroplast is about 2 mM. Such a value is not unreasonable in light of the findings that parsley carbonic anhydrase made up 1% of the total soluble protein (28).

Effect of Ethoxzolamide on Photosynthesis. Previous attempts at delineating the role of carbonic anhydrase in plants used another inhibitor—acetazolamide (10) and azide (2). It has been shown that acetazolamide inhibited photosystem II (27) and that azide did not inhibit parsley carbonic anhydrase at room temperature (28). Since acetazolamide is similar to ethoxzolamide in that both are heterocyclic sulfonamides, it is important to know whether ethoxzolamide is also inhibitory to photosynthetic electron transport. Noncyclic electron flow from H_2O to NADP (as the oxidant) was found to be insensitive to concentrations of ethoxzolamide (10-400 μM) that would have almost totally suppressed carbonic anhydrase activity (initial rates of NADP reduction were 120 μmoles hr^{-1} mg^{-1} Chl). As an indication of the effect of ethoxzolamide on photophosphorylation, electron flow was measured with coupled chloroplasts using 0.1 mM methyl viologen, ± 2 mM NH_4Cl. Control rates of O_2 uptake were around 100 μmoles hr^{-1} mg^{-1} Chl. Because NH_4Cl stimulated the rate of electron flow (generally 50–80%) while ethoxzolamide had no effect on either the rate for coupled or uncoupled chloroplasts, it was likely that ethoxzolamide at 100 μM did not affect photophosphorylation. We concluded that if this car-

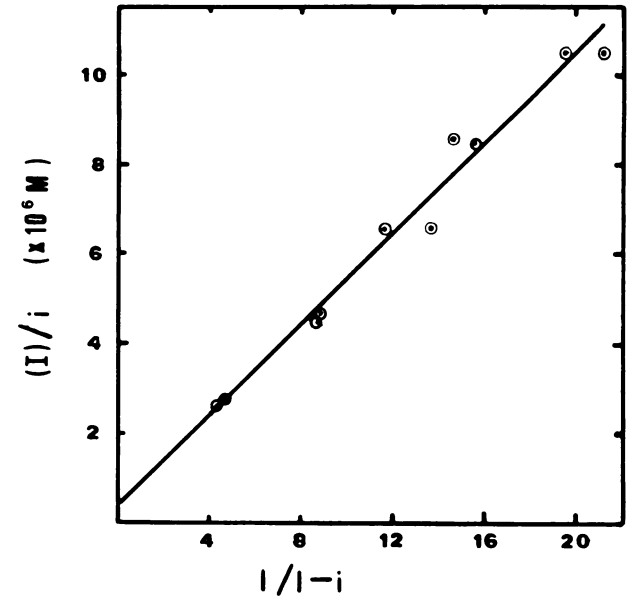

FIG. 3. Determination of carbonic anhydrase concentration by the method of Strauss and Goldstein (26). The equation for the line is $(I)/i = (Ki/1 - i) + E$. Where (I) is the ethoxzolamide concentration, Ki is the enzyme-inhibitor dissociation constant, (E) is the carbonic anhydrase concentration and i is the fractional degree of inhibition. Thus, the intercept of the curve on the $(I)/i$ axis is (E).

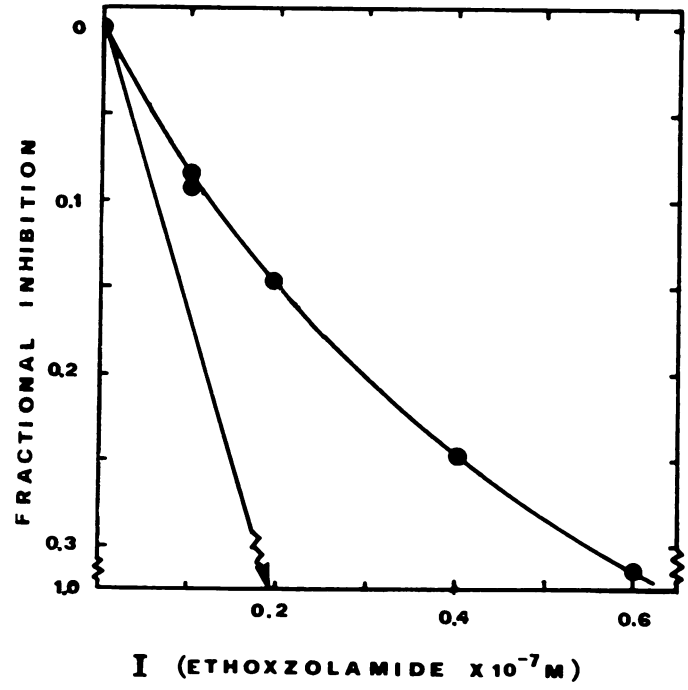

FIG. 4. Titration of carbonic anhydrase by ethoxzolamide. When F = fractional inhibition and (I) = inhibitor concentration, the equation for the curve is $F = -a(I)/[(I) + b]$. Since the dissociation constant for the enzyme-inhibitor complex is not zero, the end point for the titration of the carbonic anhydrase activity is an intercept on the (I) axis made by a line tangent to the curve at $F = 0$. Since $a = 1$, evaluating the first derivative of the equation at $F = 0$ indicates that the intercept is equal to b. A determination of b was made from a double reciprocal plot of the above curve.

Table I. *Inhibition of Photosynthetic Reduction by 100 μM Ethoxzolamide*

Intact chloroplasts were prepared and suspended as described (12). The contents of the reaction were 300 mM sorbitol, 50 mM Tricine at pH 7.6, 1.5 mM Pi, 1 mM Na-isoascorbate and, when added, 100 μM ethoxzolamide. The variation in the data is ±5%.

Oxidant Concn	Oxygen Evolution	Inhibition
mM	μmoles hr^{-1} mg^{-1} Chl	%
HCO_3^-		
1	12	43
5	19	22
10	21	19
PGA		
0.25	11	13
1	14	17
2	12	23

bonic anhydrase inhibitor depressed photosynthetic CO_2 fixation, it was not due to an inhibition of photosystems as was the case with acetozolamide.

As indicated in Table I, ethoxzolamide did, in fact, inhibit the reduction of CO_2 as measured by CO_2-dependent O_2 evolution. The inhibition was partially reversed by increasing the CO_2 concentration. These results suggested that the inhibition could have been due to the involvement of carbonic anhydrase in photosynthesis. It was also found that PGA reduction was inhibited slightly by ethoxzolamide, but with increasing concentrations of PGA the percentage of inhibition slightly increased. It is interesting to note that PGA inhibits carbonic anhydrase activity but with a K*i* of about 40 mM.

CO_2 Movement into Chloroplasts. Attempts were made at measuring the rate of CO_2 penetration into chloroplasts using the method identical to that for the measurement of acetate fluxes (13). The concentration of the total species of "CO_2" was 20 μM and the pH was 7.9. Even at the earliest time of measurement of 8 sec and at 0 C, the chloroplasts were at equilibrium with regard to "CO_2" movement. CO_2 was more concentrated in the chloroplast, thus confirming earlier and more extensive work on CO_2 binding (30). At times there was an "overshoot" of CO_2 in the chloroplast somewhat like that reported for acetate (13). Most important is the result that when 100 μM ethoxzolamide was added, the rate of "CO_2" penetration was still too fast to be detected; *i.e.* inhibition of carbonic anhydrase did not reduce the rate of "CO_2" movement such that the influx could be detected by the above method. Although it has been demonstrated previously that CO_2 movement into chloroplasts is too fast to be seen by the microcentrifuge technique, it was also found that acetazolamide, the less effective carbonic anhydrase inhibitor, reduced the rate of CO_2 penetration (30). For reasons that will be presented under "Discussion," CO_2 movement into chloroplasts is too fast to be measured by the above methods even if all of the carbonic anhydrase were inhibited. The results on inhibition by acetazolamide (30) were most likely due to an inhibition of hydration and as such the buildup of HCO_3^- but not to an inhibition of CO_2 movement.

DISCUSSION

Many suggestions have been made regarding the role of carbonic anhydrase in plants, however, none have been either substantiated or firmly ruled out. One suggestion most often put forth is the facilitation of CO_2 diffusion by the anhydrase (5, 6, 8, 10, 18). Another is that it aids in concentrating CO_2 in the chloroplast or that it causes the rapid conversion of HCO_3^- to the active species of carbon used by ribulose diphosphate carboxylase (7, 11, 21, 24, 31). In the discussion to follow, we will be able to rule out the participation of carbonic anhydrase in those reactions requiring the diffusion of CO_2 or its production from bicarbonate and support the contention that carbonic anhydrase could aid photosynthesis by transiently preventing drastic changes in pH or by binding or hydrating compounds other than CO_2.

Diffusion of CO_2. The most desirable way to rule out the proposition that carbonic anhydrase overcomes the limitation of CO_2 diffusion would be to measure the influx of CO_2 into chloroplasts with and without carbonic anhydrase activity. As indicated under "Results," the movement was too fast to be measured by contemporary procedures. Therefore, we must rely upon a more theoretical treatment and prove that the influx of CO_2 is faster than the rate of photosynthesis, or that the resistance to CO_2 in the chloroplast would be negligible when considering the other CO_2 resistances, such as those due to the stomata and mesophyll.

The nonfacilitated rate of CO_2 influx into the chloroplast can be estimated by using a modification of Fick's first law of diffusion (19): $J = P(C_o - C_i)$, where J is the rate of CO_2 movement across a unit area of chloroplast membrane, P is the permeability coefficient of CO_2, and C_o and C_i are the concentrations of CO_2 outside and inside the chloroplast, respectively. It has been pointed out that the permeability coefficient of CO_2 is greater than 10^{-2} cm sec^{-1} (19) and is most likely closer to 10^{-1} cm sec^{-1} (4). Therefore, if the concentration outside the chloroplast is that which is in equilibrium with air and that inside is negligible, J is 10^{-3} μmoles cm^{-2} sec^{-1}. For a CO_2 gradient where the concentration inside the chloroplast is maintained at no less thas 90% that outside, J is 10^{-4} μmoles cm^{-2} sec^{-1}.

What the CO_2 flux means in terms of limiting photosynthesis can be determined by calculating the rate of CO_2 movement mg^{-1} Chl hr^{-1} and demonstrating that this rate exceeds the maximum photosynthetic rate (31) of 200 to 300 μmoles CO_2 fixed mg^{-1} Chl hr^{-1}. With the knowledge that average volume of a chloroplast is 30 μm^3 (19), and that the volume of water occupied by chloroplasts making up 1 mg of Chl is 30 μl (13), the surface area occupied by the mg equivalent of chloroplasts is 10^8 cm^2, assuming that the chloroplasts are spheres. If chloroplasts are oblate spheroids or plates, the surface area is more like 1500 to 2000 cm^2 mg^{-1} Chl. From the surface area and a flux of 10^{-3} μmoles cm^{-2} sec^{-1}, the amount of CO_2 movement into the chloroplasts would be about 5000 μmoles of CO_2 mg^{-1} Chl hr^{-1}. In other words, the rate of CO_2 influx is about 20 times the maximal rate of photosynthesis. In which case, facilitating CO_2 diffusion across the chloroplast membrane by carbonic anhydrase would not increase CO_2 fixation and, therefore, contrary to previous suggestions (5, 8, 10, 11, 18, 21, 31) inhibition of fixation by carbonic anhydrase inhibitors was not due to a reduction of CO_2 influx.

Another way to analyze the effect of carbonic anhydrase on CO_2 fluxes and consequently photosynthetic carbon fixation is to consider the movement of CO_2 from the atmosphere through the inter- and intracellular spaces and finally into the chloroplast stroma where it is fixed into PGA. Such a treatment led to the suggestion that carbonic anhydrase could appreciably increase fixation of CO_2 (31). Since the time this suggestion was put forth, two important observations have been made: (*a*) all of the carbonic anhydrase in spinach is in the stroma (see "Results" and refs. 21, 30) and (*b*) CO_2 most likely moves across the chloroplast membrane, not HCO_3^-. With these observations, we will demonstrate that decreasing the resistance to

CO_2 movement in the chloroplast only would marginally increase the rate of photosynthetic CO_2 fixation.

Permeation of CO_2 can be expressed as follows: flux = force/resistivity (see ref. 19 for an excellent discussion). The total CO_2 flux (J_{CO_2}) into a photosynthesizing leaf is equal to the net or apparent rate of CO_2 fixation. Based upon a previous derivation (19), $J_{CO_2} \simeq \Delta CO_2/R_a + R_l + R_m + R_c$ for a leaf with a low percentage of CO_2 production due to respiration. Though not under consideration, high rates of photorespiration would not change the conclusions to be drawn from the above equation. ΔCO_2 is the concentration gradient and R_a, R_l, R_m and R_c are the resistances that CO_2 encounters in air, leaf, mesophyll, and chloroplast, respectively, R_c incorporates the resistances due to both membrane and stroma and is about 0.6 sec cm^{-1} when the permeability coefficient for CO_2 is 10^{-1} cm sec^{-1}. R_a, R_l, and R_m are 0.8, 3.5, and 3.5 sec cm^{-1}. If the resistance in the chloroplast were reduced to zero due to the presence of carbonic anhydrase, the total resistance met by the incoming CO_2 would be 7.8 sec cm^{-1}, in which case, J_{CO_2}, *i.e.*, net photosynthesis, would be increased by a mere 7.7% regardless of the ΔCO_2. In other words, if the carbonic anhydrase were used to facilitate CO_2 diffusion to the site of fixation, the rate of photosynthesis would be maximally increased 7.7%. The amount of carbonic anhydrase needed to reduce R_c would be much less than that which is found in the chloroplast. Though the various resistances can be altered, the conclusion would still be the same. The discussions of diffusion and resistances clearly indicated that the role of carbonic anhydrase in spinach was not to facilitate CO_2 movement.

Concentrating CO_2 by Carbonic Anhydrase. The observation that an increased CO_2 concentration increased photosynthesis fostered, in part, the suggestion that carbonic anhydrase enhanced fixation by increasing the intrachloroplastic levels of CO_2 (7, 11, 21, 24, 31). More recently it has been shown that the K*m* of CO_2 for ribulose diphosphate carboxylase was similar to the concentration of CO_2 that was in a solution which was in equilibrium with air (3). The K*m* taken together with the V_{max} also supports the concentrating effect of carbonic anhydrase since CO_2 would be in the first order region of the fixation reaction and, therefore, doubling the CO_2 concentration would nearly double the rate of its incorporation assuming nonlimiting levels of ribulose diphosphate. There are two ways in which carbonic anhydrase could function as a concentrating mechanism in the chloroplast: (*a*) carbonic anhydrase could bind CO_2 and HCO_3^- and (*b*) a high HCO_3^- pool would be maintained by carbonic anhydrase rapidly hydrating the incoming CO_2. The discussions to follow will show that whatever the concentrating mechanism, it will not enhance direct CO_2 fixation.

An estimate of the amount of CO_2 that could be concentrated by carbonic anhydrase can be determined from the K*m*, and the knowledge that the chloroplast is about 2 mM carbonic anhydrase (see Results). From the fact that $Km_{CO_2} \simeq (CO_2)(CA_{free})/(CA - CO_2)$, $Km_{HCO_3^-} \simeq (HCO_3^-)(CA_{free})/(CA - HCO_3^-)$ and that $(CA_{total}) = (CA_{free}) + (CA - HCO_3^-)$ the concentration of CO_2^- carbonic anhydrase complex can be determined by combining the above equilibrium expressions with the conservation of total enzyme equation such that $(CA - CO_2)/(CA_{total}) = [(CO_2)/Km_{CO_2}]/[1 + (CO_2)/Km_{CO_2} + (HCO_3^-)/(Km_{HCO_3^-}]$. A similar equation can be derived for the amount of bicarbonate-carbonic anhydrase complex. From a Km_{CO_2} of 29.9 mM and a $Km_{HCO_3^-}$ of 30 mM (15) and when the pH of the chloroplast is 8.2 and the carbonic anhydrase concentration is 2 mM, $(CA - CO_2) \simeq 7 \times 10^{-4}$ mM and $(CA - HCO_3^-) \simeq 7 \times 10^{-2}$ mM. If both enzyme substrate complexes were considered to be active forms for CO_2 fixation, the active CO_2 pool would be increased 7-fold and as such could markedly increase photosynthesis providing certain criteria were met.

In order for carbonic anhydrase-"CO_2" complexes to enhance fixation there would have to be a direct transfer of CO_2 from the anhydrase to the carboxylase. In addition, it must be shown that the affinity of CO_2 for carbonic anhydrase is greater than it is for ribulose diphosphate carboxylase and that the affinity of the "CO_2"-CA complex is greater than that of free CO_2 for the carboxylase. However, as has been indicated above the K*m* of CO_2 and HCO_3^- for the anhydrase is around 30 mM whereas the K*m* of CO_2 for the carboxylase is 0.011 to 0.018 mM (3). Therefore, it does not seem likely that carbonic anhydrase aids photosynthesis by first binding "CO_2" and then transferring it to ribulose diphosphate carboxylase. Furthermore, since the incoming carbon is CO_2, it would be more advantageous to bind CO_2 directly to the carboxylase, as can be suggested from the K*m*, than it would be to go through another low affinity intermediate, *i.e.*, CA-CO_2 prior to fixation.

Though it would be useless to photosynthetic CO_2 fixation to convert incoming CO_2 to HCO_3^-, it could be advantageous for carbonic anhydrase to dehydrate the bicarbonate pools to CO_2. This has been suggested by many investigators (7, 11, 21, 29, 30). Two things must be demonstrated before this suggestion can be considered valid: (*a*) the uncatalyzed rate of CO_2 formation from the HCO_3^- pool must be less than the rate of photosynthesis and (*b*) the amount of bicarbonate in the pool must be known. The operational rate constant for the dehydration of bicarbonate is about 10^{-3} sec^{-1} at a pH close to 8.0 (16). For chloroplasts at this pH and in equilibrium with the atmospheric pressure of CO_2, the HCO_3^- concentration is about 1 mM. Thus, the initial rate of dehydration would be 10^{-3} mM sec^{-1}. The dehydration rate for 1 mg Chl equivalent of chloroplasts would be on the order of 2×10^{-3} μmoles sec^{-1} which is far below the rate of CO_2 fixation. While such rates would tend to lead one to the conclusion that carbonic anhydrase would aid photosynthesis by increasing the rate of CO_2 formation, one must also ask how much it would help. The maximum amount of dissolved HCO_3^- that can be inside a chloroplast at pH 8.0 is about 0.03 μmole mg^{-1} Chl. At a rate of CO_2 fixation of 200 μmoles mg^{-1} Chl hr^{-1} there would be enough intrachloroplastic HCO_3^- to support 0.5 sec of photosynthesis. Therefore, though the noncatalyzed rate of CO_2 formation is low, it is highly doubtful that speeding up the rate with carbonic anhydrase would increase CO_2 fixation to a level that could be a significant contribution to the total amount of carbon incorporated into a spinach leaf.

For reasons similar to those given above, it is doubtful that the HCO_3^- pool would play a significant role in extensively buffering the chloroplast as has been suggested (30). Whether carbonic anhydrase would have a long range kinetic buffering effect is also doubtful since the plant could afford the longest lag time that could be imposed by the absence of the enzyme. For example, the noncatalyzed half-time for HCO_3^- titration of H^+ produced during CO_2 fixation would be 10 min—a short time when considering the total day length. Furthermore, to eliminate the lag time the chloroplast would be but 1/5000 the amount of carbonic anhydrase that has been observed.

Possible Roles for Carbonic Anhydrase. We have ruled out the need for carbonic anhydrase in aiding steady state photosynthesis. However, the anhydrase could function by mediating short term transient effects such as pH jumps that could accompany proton pumping and/or CO_2 incorporation into ribulose-1,5-diP. If the pH suddenly and drastically changed in a localized region of the chloroplast, as might be expected for

leaves intermittently shading each other, a localized denaturation of proteins could occur unless the H_3O^+ or OH^- ions could rapidly diffuse away or be converted into weaker acids and bases. Though the over-all long term buffering capacity is low, localized and immediate titrations could be maintained by rapidly catalyzing the interconversion of CO_2 and HCO_3^-. The catalyst would have to be highly concentrated such that hydroxyl ions, for example, would have a higher probability of colliding with the catalyst than other proteins. Carbonic anhydrase fits the above criteria in that it is highly concentrated in the stroma and has an exceptionally high turnover number, *i.e.* catalytic constant (see "Results"). A precedent for the protective nature of carbonic anhydrase is found in catalase—a highly concentrated enzyme with a high turnover number catalyzing the destruction of H_2O_2, a toxic oxidant.

Another function for carbonic anhydrase that seems to have eluded previous suggestions is that it may enhance photosynthetic carbon metabolism by catalyzing the hydration of aldehydes or by binding intermediates that are enzymatic effectors. Such an enzymatic activity was reported for the hydration of pyridine aldehydes (20) and we showed that PGA probably bound carbonic anhydrase since its inhibition of CA had a K_i of about 40 mM. Furthermore, it has been demonstrated that aldolase and isomerase were specific for the keto rather than the hydrated form of glyceraldehyde-3-P (23). Therefore, future investigations into the role of carbonic anhydrase should not be restricted to a study of the modulation of CO_2/HCO_3^-.

Addendum. The Km values for highly purified spinach carbonic anhydrase (see Y. Pocker and S. Y. Ng. 1973. Biochemistry 12: 5127–5134) support the conclusions drawn in our discussions.

Acknowledgments—The authors appreciate the helpful technical assistance and discussions of Mr. Jerry Cohen, Mr. Steve Hoffman, and Mr. Al Schlundt. We wish to express our gratitude to Ms. Linda Lawton for her help in the preparation of this manuscript.

LITERATURE CITED

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Avron, M. and M. Gibbs. 1974. Carbon dioxide fixation in the light and in the dark by isolated spinach chloroplasts. Plant Physiol. 53: 140–143.
3. Bahr, J. T. and R. G. Jensen. 1974. Ribulose diphosphate carboxylase from freshly ruptured spinach chloroplasts having an *in vivo* Km (CO_2). Plant Physiol. 53: 39–44.
4. Blank, M. and F. J. W. Roughton. 1960. The permeability of monolayers to carbon dioxide. Trans. Faraday Soc. 56: 1823–1841.
5. Brown, G., E. Selegny, C. Tran Minh, and D. Thomas. 1970. Facilitated transport of CO_2 across a membrane bearing carbonic anhydrase. FEBS Lett: 7: 223–226.
6. Enns, T. 1967. Facilitation by carbonic anhydrase of carbon dioxide transport. Science 155: 44–47.
7. Everson, R. G. 1970. Carbonic anhydrase and CO_2 fixation in isolated chloroplasts. Phytochemistry 9: 25–32.
8. Everson, R. G. 1971. Carbonic anhydrase in photosynthesis. *In:* M. D. Hatch, C. B. Osmond, and R. O. Slatyer, eds., Photosynthesis and Photorespiration. Wiley-Interscience, New York. pp. 275–281.
9. Everson, R. G. and C. R. Slack. 1968. Distribution of carbonic anhydrase in relation to the C_4 pathway of photosynthesis. Phytochemistry 7: 581–584.
10. Graham, D., C. A. Atkins, M. L. Reed, B. D. Patterson, and R. M. Smillie. 1971. Carbonic anhydrase, photosynthesis and light-induced pH changes. *In:* M. D. Hatch, C. B. Osmond and R. O. Slatyer, eds., Photosynthesis and Photorespiration. Wiley-Interscience, New York. pp. 267–274.
11. Graham, D. and M. L. Reed. 1971. Carbonic anhydrase and the regulation of photosynthesis. Nature 231: 81–83.
12. Jacobson, B. S., C. G. Kannangura, and P. K. Stumpf. 1973. Biosynthesis of α-linolenic acid by disrupted chloroplasts. Biochem. Biophys. Res. Commun. 51: 487–493.
13. Jacobson, B. S. and P. K. Stumpf. 1972. Fat metabolism in higher plants. LV. Acetate uptake and accumulation by class I and class II chloroplasts from *Spinacia oleracea*. Arch. Biochem. Biophys. 153: 656–663.
14. Karler, R. and D. M. Woodbury. 1960. Intracellular distribution of carbonic anhydrase. Biochem. J. 75: 538–543.
15. Kisiel, W. and G. Graf. 1972. Purification and characterization of carbonic anhydrase from *Pisum sativum*. Phytochemistry 11: 113–117.
16. Magid, E. and B. O. Turbeck. 1968. The rates of the spontaneous hydration of CO_2 and the reciprocal reaction in neutral aqueous solutions between 0° and 38°. Biochim. Biophys. Acta 165: 515–524.
17. Maren, T. H. 1967. Carbonic anhydrase: chemistry, physiology and inhibition. Physiol. Rev. 47: 595–781.
18. Nelson, E. B., A. Cenedella, and N. E. Tolbert. 1969. Carbonic anhydrase in *Chlamydomonas*. Phytochemistry 8: 2305–2306.
19. Nobel, P. S. 1974. Introduction to Biophysical Plant Physiology. W. H. Freeman, Co., San Francisco.
20. Pocker, Y. and J. E. Meany. 1967. The catalytic versatility of erythrocyte carbonic anhydrase. II. Kinetic studies of the enzyme-catalyzed hydration of pyridine aldehydes. Biochemistry 6: 239–246.
21. Poincellot, R. P. 1972. Intracellular distribution of carbonic anhydrase in spinach leaves. Biochim. Biophys. Acta 258: 637–642.
22. Randall, P. J. and D. Bouma. 1973. Zinc deficiency, carbonic anhydrase and photosynthesis in leaves of spinach. Plant Physiol. 52: 229–232.
23. Reynolds, S. J., D. W. Yates, and C. I. Poyson. 1971. Dihydroxyacetone phosphate: its structure and reactivity with α-glycerophosphate dehydrogenase, aldolase and triose phosphate isomerase and some possible metabolic implications. Biochem. J. 122: 285–297.
24. Rocha, V. and I. P. Ting. 1970. Preparation of cellular plant organelles from spinach leaves. Arch. Biochem. Biophys. 140: 398–407.
25. Smith, F. A. 1971. Transport of solutes during photosynthesis: Assessment. *In:* M. D. Hatch, C. B. Osmond and R. O. Slatyer, eds., Photosynthesis and Photorespiration. Wiley-Interscience, New York. pp. 302–305.
26. Strauss, O. H. and A. Goldstein. 1943. Zone behavior of enzymes: illustrated by the effect of dissociation constant and dilution on the system cholinesterase-physostigmine. J. Gen. Physiol. 26: 559–585.
27. Swader, J. A. and B. S. Jacobson. 1972. Acetazolamide inhibition of photosystem II in isolated spinach chloroplasts. Phytochemistry 11: 65–70.
28. Tobin, A. J. 1970. Carbonic anhydrase from parsley leaves. J. Biol. Chem. 245: 2656–2666.
29. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric Techniques. Burgess Publishing Co., Minneapolis, Minn.
30. Werden, K. and H. W. Heldt. 1972. Accumulation of bicarbonate in intact chloroplasts following a pH gradient. Biochim. Biophys. Acta 283: 430–441.
31. Zelitch, I. 1971. Photosynthesis, Photorespiration and Plant Productivity. Academic Press, New York. p. 347.