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Effect of Carbonic Anhydrase Inhibitors on Inorganic Carbon Accumulation by *Chlamydomonas reinhardtii*[1]

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ABSTRACT

Membrane-permeable and impermeable inhibitors of carbonic anhydrase have been used to assess the roles of extracellular and intracellular carbonic anhydrase on the inorganic carbon concentrating system in *Chlamydomonas reinhardtii*. Acetazolamide, ethoxzolamide, and a membrane-impermeable, dextran-bound sulfonamide were potent inhibitors of extracellular carbonic anhydrase measured with intact cells. At pH 5.1, where CO_2 is the predominant species of inorganic carbon, both acetazolamide and the dextran-bound sulfonamide had no effect on the concentration of CO_2 required for the half-maximal rate of photosynthetic O_2 evolution ($K_{0.5}[CO_2]$) or inorganic carbon accumulation. However, a more permeable inhibitor, ethoxzolamide, inhibited CO_2 fixation but increased the accumulation of inorganic carbon as compared with untreated cells. At pH 8, the $K_{0.5}(CO_2)$ was increased from 0.6 micromolar to about 2 to 3 micromolar with both acetazolamide and the dextran-bound sulfonamide, but to a higher value of 60 micromolar with ethoxzolamide. These results are consistent with the hypothesis that CO_2 is the species of inorganic carbon which crosses the plasmalemma and that extracellular carbonic anhydrase is required to replenish CO_2 from HCO_3^- at high pH. These data also implicate a role for intracellular carbonic anhydrase in the inorganic carbon accumulating system, and indicate that both acetazolamide and the dextran-bound sulfonamide inhibit only the extracellular enzyme. It is suggested that HCO_3^- transport for internal accumulation might occur at the level of the chloroplast envelope.

The green alga, *Chlamydomonas reinhardtii*, exhibits a higher affinity for C_i ($HCO_3^- + CO_2$)[2] when grown phototropically at air levels of CO_2 than when grown with air supplemented with 1 to 5% CO_2 (3). This adaptation to limiting CO_2 is correlated with increased levels of CA (6, 8) and the ability of air-grown cells to concentrate C_i internally to levels higher than could be obtained by simple diffusion (2). The $K_{0.5}(CO_2)$ of air-grown cells for photosynthesis is much lower (<1 μM) than the $K_m(CO_2)$ of ribulose-P_2 carboxylase (29–57 μM) isolated from the same organism (3, 12). In addition, air-grown cells appear to lack photorespiration, in that the cells have a low compensation point (<1 μM C_i), there is no significant O_2 inhibition of net CO_2 fixation (17), and the cells excrete little glycolate in the absence of compounds that block the C_2 pathway (22). An inorganic carbon concentrating system appears to be responsible for these photosynthetic characteristics in *Chlamydomonas* (2, 3) and other unicellular algae (7, 14), just as the C_4 cycle in higher plants concentrates CO_2 in the bundle sheath cells to suppress the oxidative photosynthetic carbon cycle.

Previous studies using inhibitors (2, 30) or genetic manipulation of algae (25), have indicated that induction of CA activity is an important part of adaptation to low CO_2 conditions. A majority of the CA activity in air-grown *Chlamydomonas* cells is located extracellularly, either in the periplasmic space or associated with the cell wall (16). In cultures of the cell wall-less mutant, CW-15, between 80 and 90% of the CA activity is released to the media (16). Recent work has indicated that this extracellular enzyme may help supply the cell with CO_2, the C_i species thought to cross the plasmalemma (18, 21, 30). In *Chlamydomonas*, the C_i accumulating system probably requires other components in addition to this extracellular CA. Spalding *et al.* (25) have evidence that a *Chlamydomonas* mutant, possibly deficient in intracellular CA, has a reduced ability to efficiently utilize C_i.

Sulfonamides are specific, high-affinity inhibitors of CA from a variety of sources (19). The sulfonamides are thought to bind near the active site of the enzyme (23). The pharmacological effectiveness of various CA inhibitors is dependent on such factors as the affinity for CA and the permeability of the inhibitor to biological membranes (19). DBS have been used to assess the role of membrane-bound forms of CA in bicarbonate resorption by kidney tubules (27). In this report, we have used a membrane-permeable sulfonamide (EZ), a soluble, membrane-impermeable sulfonamide (AZ), and a membrane-impermeable DBS to differentiate the roles of extracellular and intracellular CA in *Chlamydomonas*. Evidence is presented for the importance of intracellular CA for the cells to efficiently utilize accumulated C_i, whereas the extracellular CA (periplasmic or cell wall-associated) is primarily responsible for supplying CO_2 to the cells at alkaline pH. A preliminary report of some of this work has been presented (20).

MATERIALS AND METHODS

Measurement of Photosynthetic O_2 Evolution. *Chlamydomonas reinhardtii* strains 90 and the wall-less mutant CW 15+, from the algal collection at the University of Texas-Austin, were grown phototropically in minimal media (26) and harvested as previously described (21). All the data present in the tables and legends are with strain 90. Photosynthetic, CO_2-dependent O_2 evolution was measured with a Rank Brothers O_2 electrode (2). Harvested cells were diluted from a concentrated suspension to 25 μg Chl/ml in the buffers indicated in the Table and Figure legends. The buffers were prepared fresh daily and prior to the addition of cells bubbled with N_2 to reduce both the dissolved

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[2] Abbreviations: C_i, inorganic carbon ($CO_2 + HCO_3^-$); CA, carbonic anhydrase; AZ, acetazolamide; EZ, ethoxzolamide; DBS, dextran-bound sulfonamide; I_{50}, concentration of inhibitor required for 50% inhibition; $K_{0.5}(CO_2)$, concentration of inorganic carbon required for 50% of the maximal rate of photosynthetic oxygen evolution.

CO_2 and O_2. Cell suspensions were then illuminated with 700 $\mu E\ m^{-2}\ s^{-1}$ of 400 to 700 nm light at 25°C in the O_2 electrode chamber, until the endogenous CO_2 and HCO_3^- present was consumed as judged by the cessation of O_2 evolution (<5 min). Rates of CO_2-dependent O_2 evolution were then measured after addition of known amounts of $NaHCO_3$ or CO_2 gas to the CO_2-depleted cells. $K_{0.5}(CO_2)$ was measured as described previously (21).

Inorganic Carbon Uptake. The uptake of C_i by algal cells was estimated by silicone oil filtration (3, 10). Assays were performed in the light (400 $\mu E\ m^{-2}\ s^{-1}$) at 25°C in 400 μl microfuge tubes in a Beckman Microfuge II. The tubes contained (from bottom to top): 25 μl of either 1 M glycine (pH 10) with 0.75% (w/v) SDS or 2.5 N NaOH; 65 μl of silicone oil (1:1 (v/v) of Wacker AR20 and Wacker AR200); and 300 μl of the algal suspension that had been previously depleted of CO_2. While the cells were illuminated, $NaHCO_3$ was added to the suspension and the incubation allowed to proceed at room temperature for the indicated times. The reaction was terminated by centrifugation for 20 s in the light (21). Internal C_i was calculated from the difference between the total and the acid-stable ^{14}C in the pellet (3). The intracellular volume was calculated using [^{14}C]sorbitol and ^3H_2O (10). Chl was determined spectrophotometrically.

Carbonic Anhydrase Assays. CA activity in intact cells was measured at 4°C by adding 100 μl of a cell suspension (250 μg Chl/ml) to 5 ml 20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (Epps) pH 8.3 and the reaction was initiated by the addition of 3.4 ml ice-cold CO_2-saturated water and the time required for the pH to drop from 8.3 to 6.3 was measured. The activity was calculated by, equation units = $10[(t_0/t)-1]$, where t is the time measured for the pH change to occur when cells were present and t_0 is the time required when no cells were included. A similar control time (t_0) was measured when a previously boiled cell suspension was used in the assay, or when a large excess of a CA inhibitor was included in the assay (1 mM AZ or EZ).

Preparation of the Dextran-Bound Sulfonamide (DBS). 5-Amino-1,3,4-thiadiazole-2-sulfonamide was prepared from the acid hydrolysis of AZ as described by Kandel *et al.* (13). 5-Succinylamido-1,3,4-thiadiazole-2-sulfonamide (prepared from 5-amino-1,3,4-thiadiazole-2-sulfonamide) and aminoethyl-dextran (average mol wt, 9000) were synthesized and coupled using dicyclohexylcarbodiimide by the procedures of Tinker *et al.* (27). The DBS was separated from uncoupled ligand after the coupling reaction by exclusion from Sephadex G-10 (27). The extent of derivatization of the aminoethyl-dextran was estimated by measuring the A_{295} of the purified DBS dissolved in 50 mM NaOH using the measured ϵ of 14,638 M^{-1} for the 5-succinylamido-1,3,4-thiadiazole-2-sulfonamide. On this basis, 25 nmol of ligand was bound per mg DBS.

Glycolate Excretion. Air-grown *Chlamydomonas* were resuspended in 25 mM Na-Hepes (pH 7.3) to a cell concentration of 100 μg Chl/ml and were bubbled with air at 25°C with illumination (400 $\mu E\ m^{-2}\ s^{-1}$) for 30 min either in the absence or presence of the inhibitors indicated in Table II. At zero time and at 15 and 30 min, aliquots of cells were removed and centrifuged for 30 s in an Eppendorf centrifuge. A 200-μl aliquot of the supernatant was assayed for glycolate by the Calkins method (5).

Materials. AZ (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), sulfanilamide, bromoethylamine hydrobromide, and dextran (average mol wt, 9000) were purchased from Sigma. Dicyclohexylcarbodiimide was from Aldrich. EZ (6-ethoxy-2-benzathiazole-2-sulfonamide) was the generous gift of Dr. Thomas H. Maren. Wacker silicone oils were provided by SWS Silicones, Adrian, MI. $NaH^{14}CO_3$ was from New England Nuclear.

RESULTS

Effect of Acetazolamide and Ethoxzolamide on the Rate of Photosynthetic O_2 Evolution. When air-grown *Chlamydomonas* cells were incubated with either 75 μM CO_2 or 75 μM HCO_3^- at a given pH, there was no difference in the rate of O_2 evolution (Fig. 1, A and B) or CO_2 fixation. However, the rate that these substrates are taken up from the media was dependent on the external pH. At pH 5.1, where 94% of the C_i was present as CO_2 at equilibrium, the cells evolved O_2 at a maximal rate until the CO_2 was nearly depleted (Fig. 1A). At pH 8.0, the rate of O_2 evolution was slower (Fig. 1B). However, the calculated $K_{0.5}(CO_2)$ at both of these pH values was about the same and this has been interpreted to indicate that CO_2 is the C_i species that crosses the plasma membrane (21). The observation that added HCO_3^- also causes the same rate of O_2 evolution as does added CO_2 at each external pH can be explained by the rapid equilibration of CO_2 and HCO_3^- catalyzed by extracellular CA.

In the presence of the CA inhibitor AZ, the addition of HCO_3^- at pH 8.0 resulted in only a very slow rate of O_2 evolution (Fig. 1D). When CO_2 was added at pH 8.0 in the presence of AZ, there was initially as rapid a rate of O_2 evolution as without the CA inhibitor, but O_2 evolution quit before the added C_i was depleted. This is likely due to the slower nonenzymic equilibration of CO_2 with HCO_3- at pH 8.0 in the presence of AZ. This slow equilibration took sufficient time during which some CO_2 entered the cells resulting in the observed, rapid, O_2 evolution rate before the rest was nonenzymically hydrated and converted to HCO_3^-. That fraction of the CO_2 that was converted to HCO_3^- could not enter the cell and the rate of O_2 evolution slowed considerably even though the HCO_3^- was not depleted (Fig. 1D). This is consistent with the slow rate of O_2 evolution observed when HCO_3^- was added to the cell at pH 8.0 in the presence of the CA inhibitor AZ.

At pH 5.1, the rate of O_2 evolution was unaffected by 0.1 mM AZ (Fig. 1C). If CO_2 were the C_i species entering the cell, the uncatalyzed rate of the HCO_3^- to CO_2 conversion was rapid and complete enough at pH 5.1 to supply the cell with adequate CO_2 to allow high rates of O_2 evolution. These results agree with those

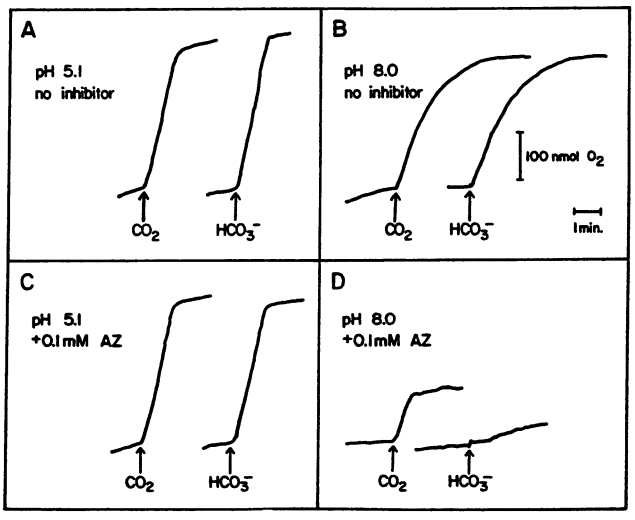

FIG. 1. Effect of AZ on the rate of O_2 evolution at pH 5.1 and 8.0. Air-grown *Chlamydomonas* cells were depleted of CO_2 in the O_2 electrode chamber in either 25 mM citrate buffer (pH 5.1) or 25 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid buffer (pH 8.0). AZ was added to 0.1 mM as indicated. Bicarbonate or CO_2 was then added to 75 μM as indicated and photosynthesis as O_2 evolution was measured.

of Tsuzuki (30) who found that the rate of CO_2 fixation in 5 s in the presence of AZ was constant and rapid from pH 6.4 to 8.9 if CO_2 was added as the substrate. However, when the C_i species was HCO_3^-, the 5-s fixation rate was very low at pH greater than 7.0 in the presence of AZ. These data are also consistent with previous evidence (18, 21, 30) which indicated that CO_2 is the C_i species that enters the cell and that the role of extracellular CA is to replenish CO_2 from the external HCO_3^- pool at alkaline pH.

While AZ had no effect on air-grown *Chlamydomonas* cells at pH 5.1, EZ caused a dramatic inhibition of O_2 evolution, even at pH 5.1, when limiting concentrations of CO_2 were supplied (Fig. 2), but no inhibition was observed at saturating levels of C_i (see below). Since at pH 5.1 extracellular CA is not necessary to supply CO_2 to the cells, these results suggest that EZ decreases the rate of O_2 evolution by affecting C_i accumulation in another manner.

Inhibition of Extracellular Carbonic Anhydrase by Carbonic Anhydrase Inhibitors. The differential inhibition of photosynthetic O_2 evolution by EZ and AZ might be explained if EZ was a more effective inhibitor of the extracellular CA than was AZ. Another possibility is that since EZ is more membrane permeable than AZ (19), EZ may enter the cell and inhibit one or more intracellular CA, as well as inhibiting the external CA. To determine the effect of AZ and EZ on the extracellular CA, its activity was measured in intact air-grown *Chlamydomonas* cells in the presence of varying concentrations of AZ and EZ. The inhibition by these two sulfonamides was also compared to the inhibition by a DBS which should be totally impermeable to the plasma membrane (Fig. 3). Both AZ and EZ inhibit CA activity at low concentrations with I_{50} values of 8×10^{-9} M and 6×10^{-9} M, respectively (Fig. 3). The I_{50} value for AZ was the same as that reported for purified CA from *Chlamydomonas reinhardtii* (4). Since the I_{50} values for these compounds were similar and very low, EZ is not simply a better inhibitor of extracellular CA. The DBS was also a potent inhibitor of extracellular CA, but the I_{50} was higher (1×10^{-7} M) than either AZ or EZ (Fig. 3). The ligand (5-succinylamido-1,3,4-thiadiazole-2-sulfonamide), not bound to the aminoethyl-dextran, had an I_{50} value of 2×10^{-8} M (not shown). The higher I_{50} for the DBS may be due to steric restraints for the interaction of the DBS with CA, or the impermeability of some higher mol wt dextrans through the cell wall (if the enzyme is in fact periplasmic), or to the binding of an inactive form of the inhibitor to the dextran during the DBS preparation.

Concentration Dependence of Carbonic Anhydrase Inhibitors Required for the Inhibition of O_2 Evolution at Limiting C_i. Since both AZ and EZ had similar effects on the extracellular CA (Fig. 3), the decrease in CO_2-dependent O_2 evolution by EZ at pH 5.1 may be because it can enter the cell and inhibit one or more internal carbonic anhydrases. At alkaline pH both AZ and EZ inhibit the extracellular CA to reduce CO_2 formation from the more abundant HCO_3^- pool. However, AZ had no effect on CO_2-dependent O_2 evolution at pH 5.1 even up to concentrations 10^5 times the I_{50} for extracellular CA. Conversely, the membrane permeable sulfonamide EZ decreased O_2 evolution at pH 5.1.

The effect of various concentrations of AZ and EZ on O_2 evolution was tested at pH 7.5, where HCO_3^- was the predominant species of C_i (94% of the total), by comparing initial rates of O_2 evolution (v_0) at limiting (50 μM) or saturating (20 mM) external C_i concentrations (Fig. 4). Neither AZ nor EZ significantly lowered the maximal rate of O_2 evolution at 20 mM HCO_3^- (Fig. 4). The concentration dependence for the inhibition of photosynthetic O_2 evolution at limiting (50 μM) C_i (Fig. 4), as compared to saturating C_i, decreased similarly for both AZ and EZ up to an inhibitor concentration of about 10^{-5} M. The decrease up to 10^{-5} M AZ or EZ can be attributed to inhibition of the external CA. At higher inhibitor concentrations, EZ decreased the rate of O_2 evolution at 50 μM HCO_3^- further while AZ caused no further decrease. The extra inhibition from EZ may be attributed to its inhibition of CA inside the cell. The concentration of AZ and EZ required to produce the initial phase of inhibition of O_2 evolution are about 100 times higher than the I_{50} values for extracellular CA shown in Figure 3. This suggests that there is a large excess of extracellular CA and that near complete inhibition of the enzyme was required before the inhibition of O_2 evolution at limiting C_i was observed.

Effect of Carbonic Anhydrase Inhibitors on $K_{0.5}(CO_2)$. The effect of CA inhibitors on the rate of photosynthetic O_2 evolution at varying C_i concentrations in shown in Figure 5. Values of $K_{0.5}(CO_2)$ calculated from these data are listed in Table I. Both DBS and AZ increased the $K_{0.5}(CO_2)$ at pH 8.0 from 0.6 μM to about 2 to 3 μM, but had no effect at pH 5.1 (Fig. 5, Table I). Neither AZ nor DBS had a significant effect on the maximal rate of O_2 evolution observed at saturating concentrations of C_i. Again, these data are consistent with inhibition of the extracellular CA for replenishing CO_2 from HCO_3^- at alkaline pH. The

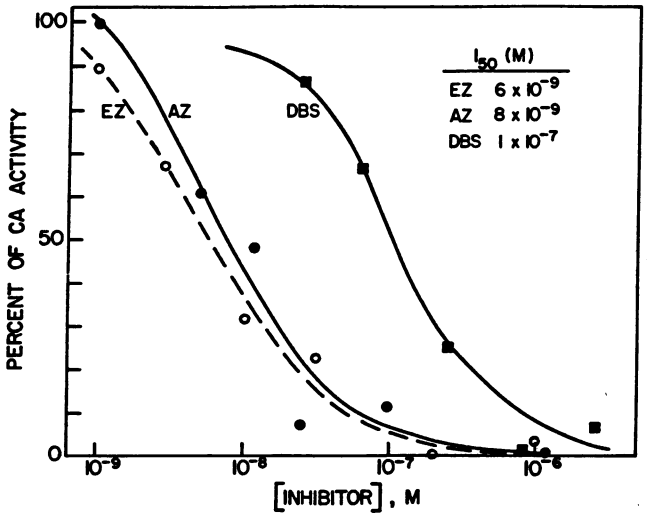

FIG. 3. Effect of carbonic anhydrase inhibitor concentrations on CA activity in intact *Chlamydomonas reinhardtii*. CA activity in whole cells was determined with varying concentrations of (○) EZ, (●) AZ, (■) or DBS. The concentrations of DBS shown are based on the concentration of the bound ligand. CA activity was 200 units/mg Chl in the absence of inhibitors.

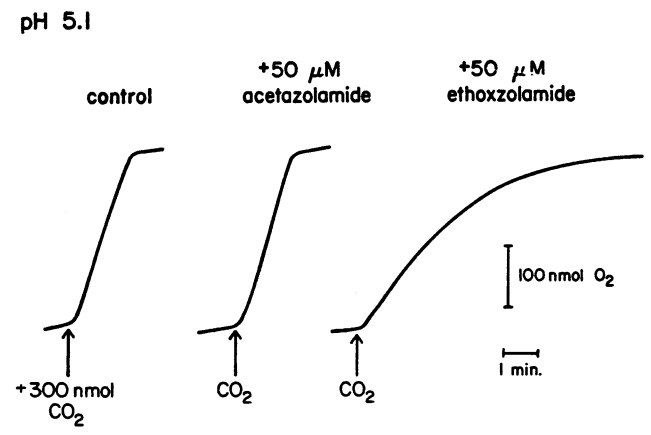

FIG. 2. Effect of AZ and EZ on O_2 evolution at pH 5.1. Air-grown *Chlamydomonas reinhardtii* cells were depleted of CO_2 in 25 mM citrate buffer (pH 5.1). AZ or EZ were then added to 50 μM as indicated. CO_2 was then added to a concentration of 75 μM and the resulting O_2 evolved was measured.

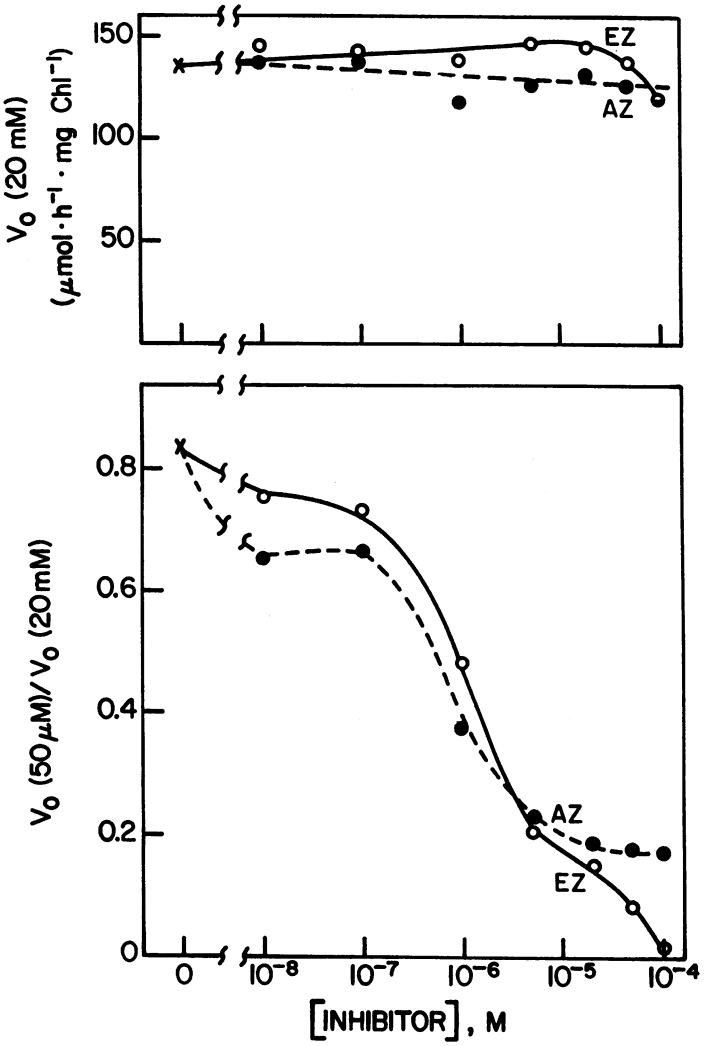

FIG. 4. Effect of varying concentrations of carbonic anhydrase inhibitors on the initial rates of photosynthetic O_2 evolution at limiting or saturating HCO_3^- conditions. The initial rates of O_2 evolution (v_0) were determined with cells in 25 mM Hepes (pH 7.5) following the addition of either 50 μM or 20 mM HCO_3^- in the presence of varying concentrations of either EZ (○) or AZ (●).

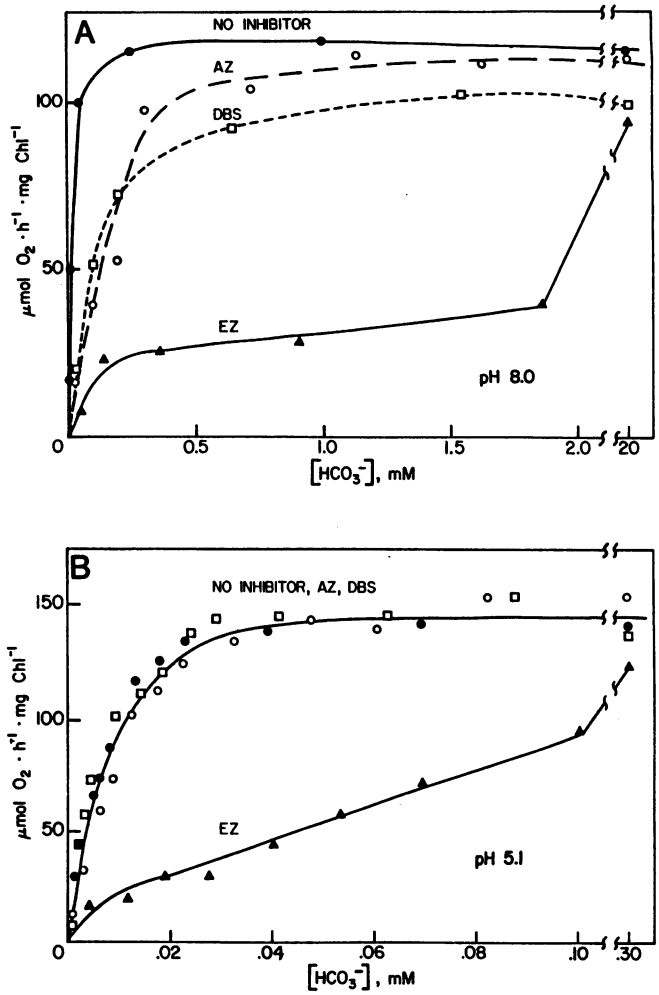

FIG. 5. Effect of CA inhibitors on the rates of photosynthetic O_2 evolution at varying concentrations of bicarbonate. The initial rates of photosynthetic O_2 evolution at different concentrations of HCO_3^- were determined in the presence of either (●) no inhibitor, (○) 50 μM AZ, (□) 330 mg/ml DBS, or (▲) 50 μM EZ in either 25 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (pH 8.0) (A) or 25 mM citrate (pH 5.1) (B).

fact that AZ increased the $K_{0.5}(CO_2)$ in the same manner as did DBS supports the presumption that AZ is not permeable to the cells.

A similar situation was observed with well-washed CW 15+ cells, a wall-less mutant of *Chlamydomonas*. With this cell line, extracellular CA can be washed away (no detectable activity) and as expected the $K_{0.5}(CO_2)$ at pH 8.0 is higher than wild-type cells (2 μM *versus* 0.4 μM, respectively). However, when 50 μM AZ was added to these wall-less cells the $K_{0.5}(CO_2)$ increased even further to 5 μM (data not shown). This implies that some extracellular CA is probably present despite the inability to detect it by the CA assays. At pH 5.1, the $K_{0.5}(CO_2)$ is 3 μM, the same as in wild-type cells, again showing that washing away the extracellular CA has no effect on C_i accumulation at this low pH. These cells also exhibit a constant $K_{0.5}(CO_2)$ of about 2 to 3 μM between pH 4.5 and 9.5 (data not shown) and concentrate CO_2 from the media (18). A similar approach was used recently by Aizawa and Miyachi (1) with *Dunaliella tertiolecta*. They removed external CA by the use of subtilisin and also observed an increase in the $K_{0.5}(CO_2)$ at pH 8.0. In this case, the protease removed all of the extracellular CA but only increased the $K_{0.5}(CO_2)$ by 3-fold. This is similar to our results with AZ and DBS and points out that the extracellular CA, while important in the adaptation to low CO_2, probably is not required to concentrate CO_2.

Table I. *Effect of Carbonic Anhydrase Inhibitors on the $K_{0.5}$ (CO_2) by Air-Grown C. reinhardtii at either pH 5.1 or 8.0*

The procedures were as described in the legend to Figure 5.

Inhibitor	pH 5.1	pH 8.0
	$K_{0.5}$ (CO_2), μM	
None	5	0.6
AZ	8	3.4
DBS	5	2.2
EZ	60	60

At both pH 5.1 and 8.0, EZ increased the $K_{0.5}(CO_2)$ in air-grown cells from 5 μM or less to about 60 μM which is similar to the $K_{0.5}(CO_2)$ measured in cells grown with 5% CO_2 that do not possess a C_i-accumulating system (3, 21). This value is also similar to the $K_m(CO_2)$ for ribulose-P_2 carboxylase from *Chlamydomonas* when measured at air levels of O_2 (3, 12). In the presence of AZ or DBS the $K_{0.5}(CO_2)$ is almost constant from pH 5 to 8.5, but never increased to the value of about 60 μM

observed with 5% CO_2-grown cells (3, 21) or air-grown cells treated with EZ. The high 60 μM value for $K_{0.5}(CO_2)$ should occur solely from CO_2 diffusion into the cell to ribulose-P_2 carboxylase in the absence or as a result of inhibition of C_i accumulation, and reflects the $K_m(CO_2)$ of ribulose-P_2 carboxylase.

Effect of Carbonic Anhydrase Inhibitors on Inorganic Carbon Uptake. At pH 5.1, AZ and DBS caused only a small decrease in $^{14}CO_2$ fixation (Fig. 6A) and no change in C_i accumulation by air-grown *Chlamydomonas* cells (Fig. 6B). EZ, on the other hand, inhibited $^{14}CO_2$ fixation by about 80% and caused a 3- to 4-fold increase in C_i accumulation within the cells (Fig. 6B). These results are similar to those obtained previously by Spalding *et al.* (25) who found an increase in C_i accumulation in cells treated with EZ and with a *Chlamydomonas* mutant that may be deficient in CA. These results and the recent measurements of CA activity by Spalding (personal communication) indicate that the mutant is likely to be deficient in an internal CA.

At pH 8.0, both AZ and EZ lowered CO_2 fixation and inhibited the accumulation of C_i when very low concentrations of C_i were present (an equilibrium CO_2 concentration of less than 1 μM) (Fig. 7). In this case, since the extracellular CA is presumably required to replenish CO_2 from the HCO_3^- pool at alkaline pH, the availability of external CO_2 probably was the limiting factor. However, when the pH was lower or the added $H^{14}CO_3^-$ higher, the decrease in C_i accumulation caused by AZ was less (data not shown) and EZ caused an increase in C_i accumulation as observed at pH 5.1 (Fig. 6B).

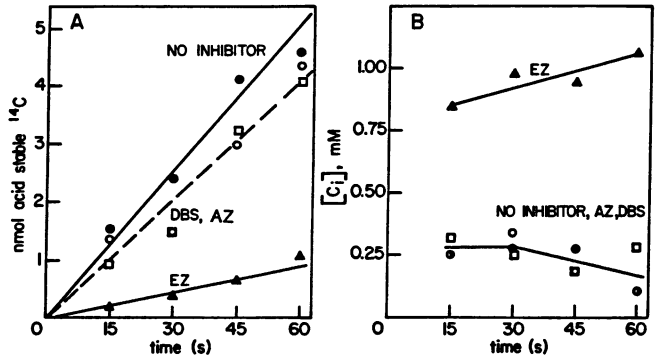

FIG. 6. Effect of CA inhibitors on $^{14}CO_2$ fixation and intracellular C_i at pH 5.1. Fixed $^{14}CO_2$ (A) and the intracellular C_i (B) were determined as described following the addition of 20 μM HCO_3^- in the presence of either (●) no inhibitor, (○) 50 μM AZ, (□) 250 mg DBS/ml, or (▲) 50 μM EZ in 25 mM citrate (pH 5.1).

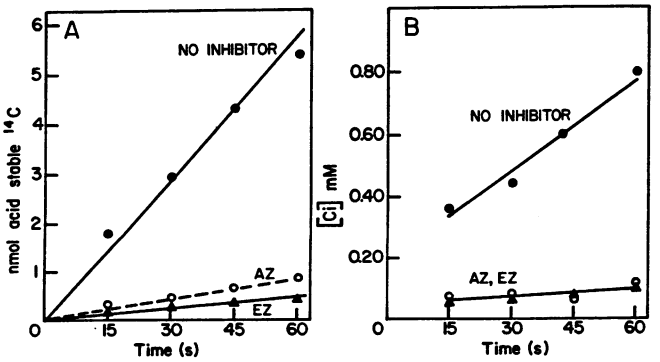

FIG. 7. Effect of CA inhibitors on CO_2 fixation and intracellular C_i accumulation at pH 8.3. Fixed CO_2 (A) and intracellular C_i (B) were determined following the addition of 40 μM HCO_3^- in the presence of either (●) no inhibitor, (○) 50 μM AZ, or (▲) 50 μM EZ in 25 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (pH 8.3).

Effect of Acetazolamide and Ethoxzolamide on Glycolate Excretion. From the above data, it appears that EZ inhibits an intracellular CA intimately linked with low CO_2 adaptation in algae. By increasing the intracellular CO_2 concentration this adaptation is thought to decrease ribulose-P_2 oxygenase activity for P-glycolate snythesis (3, 15, 29). This theory was tested by measuring glycolate excretion by air-grown cells treated with either AZ or EZ (Table II). These measurements were made both in the absence and in the presence of aminooxyacetate which effectively blocked the C_2 pathway in *Chlamydomonas*, and thus the excreted glycolate would be a measurement of the total amount of glycolate biosynthesized and an estimate of ribulose-P_2 oxygenase activity (unpublished).

When air-grown *Chlamydomonas* cells were bubbled with air at pH 7.5, neither control cells nor AZ-treated cells excreted significant amounts of glycolate in the absence of aminooxyacetate (Table II). However, when the C_2-pathway was blocked with aminooxyacetate, similar amounts of glycolate were excreted with or without AZ. The relatively low level of glycolate excretion, even with AZ inhibition of external CA at pH 7.5 where HCO_3^- predominates, can be explained by the fact that the cells were continuously bubbled with air levels of CO_2 so that the CO_2 concentration in the media remained nearly constant and was not depleted by the cells. EZ treatment caused the cells to excrete glycolate and aminooxyacetate enhanced this excretion (Table II). The excretion of glycolate by EZ-treated cells was similar to that for CO_2-grown cells with no CA or C_i accumulation system when tested at this low CO_2 level (28, 29). These results and those in Figure 6, indicate that EZ treatment eliminated a part of the C_i accumulation system involved in concentrating available CO_2 at the site of the ribulose-P_2 carboxylase/oxygenase.

DISCUSSION

The induction of CA activity is associated with the adaptation to low CO_2 conditions by *Chlamydomonas* (6, 8). However, the effect of CA inhibitors on the CO_2 concentrating mechanism has varied in different reports. In some cases, CA inhibitors caused a decrease in C_i accumulation (2) while in others they caused an increase (25). Similarly, AZ has been reported to decrease CO_2 fixation in some cases (3) but not in others (30). In this report, we have shown that AZ and EZ do not lower CO_2-dependent O_2 evolution in the same fashion, and have different effects on C_i accumulation. The presence of CA in two or more compartments that are differentially inhibited by these compounds can explain these results and the discrepancies in the literature.

These differences are likely due to the ability of EZ to penetrate biological membranes while AZ is only weakly permeable (19). Since both compounds inhibit extracellular *Chlamydomonas* CA to a similar extent (Fig. 3), the observed differences are not due to EZ inhibiting extracellular CA more effectively than AZ. Instead, the inhibition of CO_2-dependent O_2 evolution at limiting CO_2 by these two compounds (Fig. 4), implies that EZ also

Table II. *Effect of Carbonic Anhydrase Inhibitors of Glycolate Excretion in Air-Grown Chlamydomonas Cells*

Cells were illuminated in 25 mM Hepes (pH 7.5) in the presence of the indicated inhibitors. The results shown are the average ± SD for three independent experiments. The amount of glycolate excreted was measured every 15 min as previously described (5).

Inhibitor	Rate of Glycolate Excretion	
	No aminooxyacetate	+ 2 mM aminooxyacetate
	$\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl	
None	0	1.5 ± 0.6
50 μM AZ	0	1.4 ± 0.2
50 μM EZ	2.5 ± 1.2	3.6 ± 1.5

inhibits another CA, probably located within the cell. This situation may be analogous to the case in red blood cells where EZ inhibits the intracellular CA with a half-time 170 times faster than AZ (19). In support of the concept that AZ is impermeable to the cells, a DBS which is impermeable to membranes, affected C_i uptake and O_2 evolution in the same manner as AZ.

Since AZ and DBS apparently only inhibited the extracellular CA, its role in the C_i accumulation system could be assessed. The results presented here and previously (11, 18, 21, 30) have indicated that CO_2 is the C_i species that crosses the plasmalemma and therefore the role of the extracellular CA is to replenish the cells with CO_2 from external HCO_3^- (21). By doing so, the potentially rate-limiting diffusion of CO_2 across an unstirred layer (21, 24) is reduced, since at basic pH, HCO_3^- is present in higher concentrations than CO_2. The inhibition of CO_2 fixation and C_i uptake by AZ observed at high external pH is due to limiting the availability of CO_2 to the air-grown cells and not due to an inhibition of the C_i accumulating mechanism itself. It does not appear that AZ is affecting the C_i accumulating system directly because at pH 5.1, where there is abundant CO_2, AZ had no effect on air-grown cells.

Nearly 100 times as much AZ is required to decrease CO_2 fixation as is required to inhibit by 95% the external CA. This indicates that the external CA is present in excess and that a small fraction of the CA activity is sufficient to supply the cell with CO_2. In the presence of 10^{-7} M AZ for example, only a trace of external CA activity was present (Fig. 3) but CO_2 fixation and C_i accumulation were unaffected by this concentration of AZ (Fig. 4). This implies that some extracellular CA was probably present despite the inability to detect it by the CA assays. We feel it is dangerous to conclude from CA assays alone that all of the external CA has been inhibited or removed. This might also present a problem to investigators working with other algal strains that have less external CA than does *Chlamydomonas*. In those cases, a low amount of a physiologically important extracellular CA might go undetected. This could lead to errors in interpreting data as to which inorganic carbon species is taken up. The combination of CA assays and use of low concentrations of an impermeable inhibitor such as AZ or DBS can be used to determine whether extracellular CA is important in supplying the cell with CO_2.

The role of the intracellular CA(s) is not yet clear. One possible location of this CA is the chloroplast. This location is consistent with the observed increase in intracellular C_i when cells are treated with EZ (Fig. 6). An outline of one possible mechanism for C_i accumulation is given in Figure 8. In this model, CO_2 enters the cell and is hydrated to HCO_3^- in the cytoplasm, possibly by a CA located there. Our observation that a marked accumulation of C_i occurs in the presence of EZ suggests that this hydration could occur at a sufficient rate nonenzymically. The accumulation step could then be accomplished by a transporter with a high affinity for HCO_3^- located on the chloroplast envelope to increase the HCO_3^- in the stroma to levels higher than could be obtained by passive diffusion. Finally another CA located in the chloroplast stroma catalyzes the conversion of HCO_3^- to CO_2, the species of C_i utilized by ribulose-P_2 carboxylase. In this model, the higher C_i concentration would be in the chloroplast stroma where the carboxylation step takes place. Since the active site concentration of ribulose-P_2 carboxylase is high, the CO_2 present in this compartment would be likely to react with ribulose-P_2 instead of diffusing out of the chloroplast. Another possibility would be that the accumulation step takes place at the plasmalemma as has been proposed (18). In this case however, CO_2 diffusion out of the cell might present a serious problem since CO_2 is highly permeable to biological membranes (9). For this mechanism to be plausible, an EZ-insensitive CA would need be associated with the transport or be present in the cytoplasm, to allow CO_2 conversion to an impermeable form of C_i (HCO_3^-) which could accumulate intracellularly.

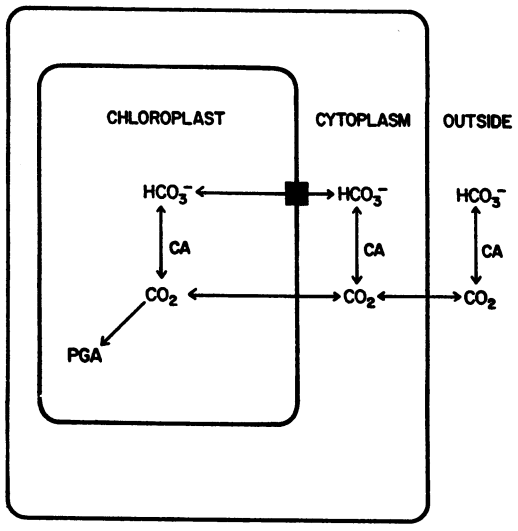

FIG. 8. A model for the accumulation of C_i by *C. reinhardtii*.

At this stage, the HCO_3^- transporter has not been directly identified or located in the cell. If both a cytoplasmic CA and a chloroplastic CA were involved in this process, the effects of EZ on C_i uptake would be expected to be complex. However, the fact that EZ inhibits CO_2 fixation at acidic external pH implies that CA is an integral part of the accumulation process. It also supports the idea that added CO_2 is converted to HCO_3^- at some stage in the accumulation process. This is because CO_2 is entering the cell and CO_2 is the form of C_i utilized by ribulose-P_2 carboxylase. If CO_2 simply diffused to the carboxylating site then inhibition of fixation by EZ would not be expected. This is the situation seen at high concentrations of CO_2 where EZ has no effect on CO_2 fixation.

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