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OXIDATIVE PHOTOSYNTHETIC CARBON CYCLE IN GREEN ALGAE

By

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ABSTRACT

OXIDATIVE PHOTOSYNTHETIC CARBON CYCLE IN GREEN ALGAE

BY

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A survey of some green algae for the type of glycolate oxidizing enzyme, the presence of peroxisomes, and the presence of a CO_2 concentrating mechanism is presented. One group of algae has glycolate dehydrogenase and one group has glycolate oxidase. Catalase is present in all of the algae studied, but in much greater amounts in the algae with glycolate oxidase.

The presence of a CO_2 concentrating mechanism was also determined in these algae by measuring the $K_{0.5}(CO_2)$ for photosynthesis and the level of external carbonic anhydrase. Some of the algae have characteristics of a CO_2 concentrating mechanism and some do not.

The CO_2 concentrating mechanism of Chlamydomonas moewusii was studied in more detail. The species of inorganic carbon taken into the cell is CO_2, and this alga lacks an external carbonic anhydrase. Photosynthetic characteristics of Chlamydomonas moewusii were compared to Chlamydomonas reinhardtii, which also takes up CO_2 but has an external carbonic anhydrase. It was found that these two algae have the same low $K_{0.5}(CO_2)$ and accumulate HCO_3^- internally to the same high level.

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LIST OF ABBREVIATIONS

AOA	Aminooxyacetate
CA	Carbonic anhydrase
Ci	Inorganic carbon
DAB	3,3'-diaminobenzidine
DCPIP	Dichloropnenolindophenol
FMN	Flavin mononucleotide
PVP	Polyvinylpyrrolidine
RUBISCO	Ribulose bisphosphate carboxylase/oxygenase

INTRODUCTION

The C_2 oxidative photosynthetic carbon cycle has been studied extensively in green algae and higher plants. Most aspects of the C_2 cycle are the same in algae and plants, however, major differences exist in the nature and location of the glycolate oxidizing enzymes. Plants and some higher algae have a glycolate oxidase located in the peroxisomes. The electron acceptor is oxygen, and its peroxisomal location with catalase is linked with detoxification of the H_2O_2 generated by this oxidase. Many algae have a mitochondrial glycolate dehydrogenase whose primary electron acceptor is unknown, although it is not oxygen. Glycolate oxidase is not inhibited by cyanide and also it oxidizes L-lactate but not D-lactate, while glycolate dehydrogenase is cyanide sensitive and oxidizes D but not L-lactate.

The location and type of the glycolate oxidizing enzyme represents a difference between eukaryotic single cell algae and higher plants and algae. Glycolate dehydrogenase has very low activity, especially when grown on high levels of CO_2, and because of this, many unicellular algae excrete much of the glycolate formed. Plants and algae with glycolate oxidase, which is much more active, do not excrete glycolate. Thus, carbon is lost by algae that excrete glycolate. Plants and algae with glycolate oxidase also lose carbon through photorespiration (theoretically 1 out of 4 carbons) but are able to recycle most of the carbon when two glycolates are metabolised to one glycerate. Since glycolate dehydrogenase is located in the mitochondria, glycolate oxidation could be linked to energy production via the electron transport chain (10,17, 55). Glycolate oxidation in the peroxisomes is not linked to electron

transport and therefore is an energy wasting process. Many algae apparently contain a few small unspecialized peroxisomes, or microbodies, which contain catalase but not glycolate oxidase.

The formation and metabolism of glycolate is dependent on the levels of O_2 and CO_2. Since glycolate is formed by the oxygenase reaction of ribulose bisphosphate carboxylase/oxygenase, the lower the CO_2/O_2 ratio the more glycolate is made. The amount of glycolate dehydrogenase may be increased by growth on low levels of CO_2 because of substrate activation.

Some plants and algae have developed ways to reduce glycolate formation. C_2 plants reduce the competition between CO_2 and O_2 by fixing CO_2 as HCO_3^- into malate and aspartate, and then decarboxylating these acids at the site of ribulose bisphosphate carboxylase, thus increasing the CO_2 concentration above levels obtained by gas diffusion into the chloroplasts. C_3 plants have no such means to reduce glycolate formation, which is apparently dependent only on atmospheric levels of CO_2 and O_2.

Some unicellular algae have also developed a CO_2 concentrating mechanism to increase CO_2 fixation and reduce photorespiration. The details for this mechanism are unknown but is thought to involve carbonic anhydrase and a HCO_3^- transporter on either the plasma membrane or the chloroplast envelope (32).

Since it thought that plants may have evolved from the green algae, changes in glycolate metabolism and the development of leaf-type peroxisomes also may have occurred during evolution. Many questions can be asked about these changes namely, why did plants and higher algae develop a wasteful glycolate oxidase instead of keeping the energy

conserving glycolate dehydrogenase; are there advantages to having a particular form of the enzyme; what environmental factors caused the change; and are all algae able to reduce photorespiration with a CO_2 concentrating mechanism?

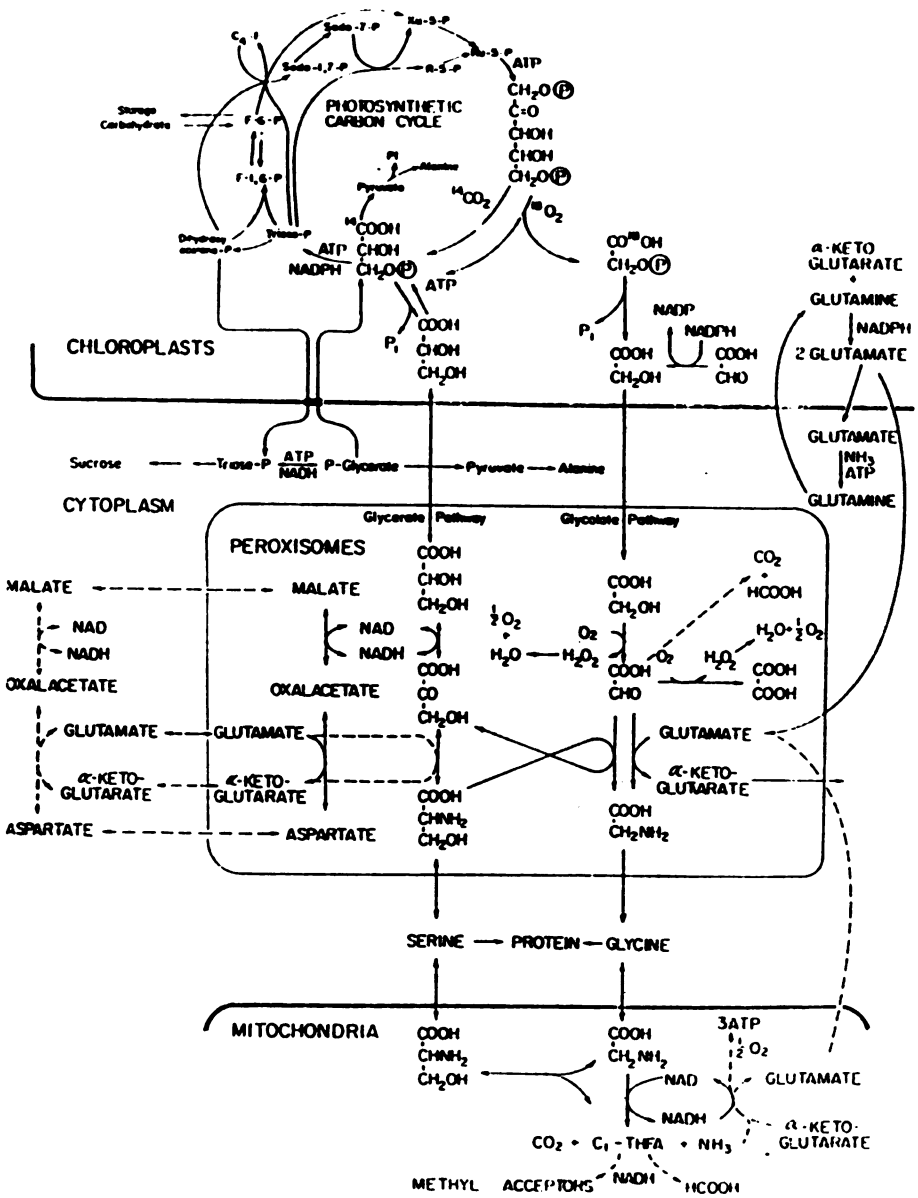
A survey of some green algae for the type of glycolate oxidizing enzyme and the presence of a CO_2 concentrating mechanism is presented in Part 1 of this thesis. During the course of this research it was discovered that Chlamydomonas moewusii has a modified CO_2 concentrating mechanism relative to Chlamydomonas reinnardtii. A more detailed investigation of Chlamydomonas moewusii is presented in Part 2.

LITERATURE REVIEW

The C_2 Cycle: The light-dependent uptake of O_2 and release of CO_2 during photosynthesis is called photorespiration. The metabolic pathway for photorespiration is known as the oxidative photosynthetic carbon cycle or C_2 cycle. The reductive photosynthetic carbon cycle, or C_3 cycle, is initiated by ribulose bisphosphate carboxylase/oxygenase (RUBISCO), and CO_2 is reduced to sugar phosphates in the chloroplast. The sugar phosphates are converted to starch for storage or transport out of the chloroplast for energy and sucrose synthesis. The C_2 and C_2 cycles are linked due to the competition between CO_2 and O_2 for RUBISCO; RUBISCO either converts CO_2 and ribulose bisphosphate to two molecules of PGA or reacts with O_2 to form one molecule of PGA and one molecule of phosphoglycolate for the C_2 cycle.

A diagram of the C_2 cycle in higher plants is shown in Figure 1 (18). The first step is the formation of P-glycolate from ribulose bisphosphate and O_2 by the oxygenase reaction of RUBISCO in the chloroplasts. P-glycolate phosphatase hydrolyzes the P-glycolate to glycolate which is transported from the chloroplasts to the peroxisomes. glycolate is oxidized in the peroxisomes to glyoxylate and H_2O_2 by glycolate oxidase. The H_2O_2 is degraded by catalase and the glyoxylate is transaminated to glycine, which is transported to the mitochondria. In the mitochondria, glycine is oxidized by glycine oxidase to CO_2, NH_3, and a C_1 unit bound to tetrahydrofolate. The C_1 unit is added to a second glycine molecule to form serine by serine-hydroxymethyl transferase. Serine is converted to hydroxypyruvate by an aminotransferase in the peroxisome, and hydroxypyruvate is reduced to glycerate by

Figure 1. The Oxidative Photosynthetic Carbon Cycle In Higher Plants. From reference 18.


PHOTOSYNTHETIC CARBON CYCLE
ATP
NADPH
CHLOROPLASTS
NADP
NADPH
α-KETO GLUTARATE
GLUTAMINE
NADPH
2 GLUTAMATE
GLUTAMATE
NH3
ATP
GLUTAMINE
Sucrose
Triose-P
ATP
NADH
P-Glycerate
Pyruvate
Alanine
CYTOPLASM
Glycerate Pathway
Glycolate Pathway
PEROXISOMES
MALATE
NAD
NADH
OXALACETATE
GLUTAMATE
α-KETO-GLUTARATE
ASPARTATE
HCOOH
SERINE
PROTEIN
GLYCINE
MITOCHONDRIA
3ATP
NAD
NADH
GLUTAMATE
α-KETO-GLUTARATE
NADH
HCOOH
METHYL ACCEPTORS

hydroxypyruvate reductase. The glycerate can return to the chloroplast where it is phosphorylated and can reenter the C_3 cycle and regenerate ribulose bisphosphate.

O_2 uptake occurs during the formation of P-glycolate by the oxygenase reaction of RUBISCO, during glycolate oxidation in the peroxisomes, and during glycine oxidation in the mitochondria. CO_2 is released during glycine oxidation.

The C_2 cycle in green algae is very similar to the cycle in plants, but has some differences. Many unicellular and multicellular algae have a mitochondrial glycolate dehydrogenase instead of a peroxisomal glycolate oxidase. The activities of glycolate dehydrogenase and glycine decarboxylase appear to be too low to account for the measured flow of carbon through the C_2 cycle (18). Cells grown on high CO_2, or treated with aminooxyacetate (AOA) which blocks conversion of glyoxylate to glycine, accumulate and excrete glycolate (50).

Photorespiration in cyanobacteria is not well understood. Many of the C_2 cycle enzymes are absent or detected only in trace levels. RUBISCO from cyanobacteria exhibits oxygenase activity, and much P-glycolate phosphatase activity is present. In fact, P-glycolate phosphatase is up to 6 times more active in cyanobacteria than green algae (19). Thus, glycolate is probably made but its metabolism is unknown. No glycolate excretion is seen even in the presence of AOA, and little if any glycolate dehydrogenase activity is detectable.

The function of the C_2 cycle is a subject of continuing investigation. It is considered a wasteful energy burning process that is unavoidable due to the oxygenase reaction of RUBISCO. The C_2 cycle can serve as a carbon scavenging system since some of the carbon atoms

flowing through the system reenter the C_3 cycle, and some of the released CO_2 can be refixed by the cell. Because the C_2 cycle can dispose of excess photosyntnetic assimilatory capacity, it may prevent photooxidative damage, especially during periods of CO_2 shortage. Another function is to provide glycine and serine and C_1 units for cell growth.

Algal Glycolate Oxidizing Enzymes: The distribution of glycolate oxidizing enzymes was studied by Frederick et al (14). They assayed several green algae, bryophytes, ferns, and fern allies for the presence of glycolate oxidase or dehydrogenase, the specificity for D or L-lactate, and the peroxisomal marker, catalase. All of the plants and a few of the algae had glycolate oxidase, but most of the algae had glycolate dehydrogenase. Glycolate oxidase also oxidized L-lactate and occasionally low amounts of D-lactate, while glycolate dehydrogenase oxidized D-lactate and low levels, if any, of L-lactate. Catalase was present in most organisms, but was usually present in much higher levels when glycolate oxidase was present.

Tolbert (51) surveyed several marine green algae and land plants for the type of glycolate oxidizing enzyme. All of the algae had glycolate dehydrogenase. The land plants had glycolate oxidase except for Cymodocea rotundata and Thallassia hemprichii, which had glycolate denydrogenase. These are the only land plants known to have glycolate denydrogenase.

In a survey of 27 species of green algae representing 16 genera in the Chlorococcolales and Chlorosarcinales, Bullock et al (7) found all but two of the species to contain glycolate dehydrogenase. Some of the species with glycolate dehydrogenase had unusually high rates of

L-lactate oxidation, 50-138% of the glycolate activity. However, activity with D-lactate was always higher than with L-lactate in these algae. One of the algae with glycolate oxidase, Planophila terrestris, also had high rates of D-lactate oxidation, 108% of glycolate oxidation, although L-lactate oxidation was much higher. Growth conditions of some of these algae were varied to see if a change in the form of enzyme occured. The type of glycolate oxidizing enzyme did not change when grown on solid or liquid media, increased nitrogen, or when grown heterotrophically or autotrophically.

Algal Peroxisomes: Organelles similar to animal and plant peroxisomes have been detected in many green algae (38). These structures are usually termed microbodies because the enzyme content is not known for most of the algae. Almost all algae studied by electron microscopy have revealed the presence of microbodies.

Silverberg (38) studied 29 species of algae by electron microscopy and found microbodies in most of them. A cytochemical stain, DAB, for catalase was performed on selected algae and was positive for the majority. Several other species have been stained with DAB by other researchers (39,48) with both positive and negative results. Silverburg states that negative findings in a cytochemical preparation do not necessarily mean the absence of enzyme activity, but may be explained by inactivation by the fixative, solubilization of the enzyme during preparation, or inadequacy of the incubation. Many algae, and other tissue, once reacting negatively with DAB, later gave a positive reaction. Thus, it is likely that all green algae have catalase-containing unspecialized microbodies regardless of which type of glycolate oxidizing enzyme is present.

Glycolate dehydrogenase has been localized in the mitochondria of a few green algae and diatoms. Using a cytochemical assay for glycolate dehydrogenase, Beezley et al (5) found deposition of a electron opaque material in the outer compartment of the mitochondria of Chlamydomonas reinhardtii. D-lactate gave similar results as glycolate, while L-lactate gave only a weak reaction. Oxamate, which inhibits glycolate dehydrogenase in cell free extracts, inhibited the cytochemical reaction. Other cellular components did not accumulate stain.

Intact organelles of a few green algae have been isolated and their enzymology characterized. Organelles from only three algae, Eremosphaera, Chlorogonium, and Euglena, with glycolate dehydrogenase have been characterized. Intact microbodies and mitochondria of Eremosphaera were isolated by Stabenau (41). The microbodies contained catalase and uricase, while the mitochondria contained the glycolate pathway enzymes, including glycolate dehydrogenase, glutamate-glyoxylate aminotransferase, serine-hydroxymethyl transferase, serine-glyoxylate amino transferase, and hydroxypyruvate reductase.

The microbodies of Chlorogonium were also isolated (46) and only catalase and uricase were present. This alga has glycolate dehydrogenase which is probably in the mitochondria, although the mitochondrial enzymes were not assayed in this study.

Euglena represents a more complex distribution of enzymes. Catalase has not been found in the microbodies, but a glycolate dehydrogenase, hydroxypyruvate reductase, and serine-glyoxylate aminotransferase are in the microbodies. Glycolate dehydrogenase was also found in an equal amount in the mitochondria along with glutamate-glyoxylate aminotransferase, glycine oxidase, and serine-hydroxymethyl

aminotransferase (11,26). The two glycolate dehydrogenases are under different physiological control, both are present when the algae are grown on air levels of CO_2, but the microbody enzyme disappears when grown on 5% CO_2 (55). The peroxisomal glycolate oxidizing system did not take up oxygen and was cyanide sensitive, typical of glycolate dehydrogenase.

Glycolate dehydrogenase was found in isolated mitochondria of two marine diatoms (36). The enzyme was shown to link indirectly to oxygen via the electron transport system.

Glycolate oxidase has been found in the peroxisomes of all plants studied, and in a few green algae. A cytochemical assay showed glycolate and L-lactate dependent reaction product in the microbodies of Klebsormidium (15). These microbodies also stained positive for catalase (48).

Intact peroxisomes and mitochondria were isolated from Spirogyra (45). Glycolate oxidase, catalase, and hydroxypyruvate reductase were found in the microbodies, other enzymes were not looked for. Microbodies of Mougeotia were also isolated (43), and found to contain catalase, glycolate oxidase, alanine-glyoxylate aminotransferase, glutamate-glyoxylate aminotransferase, and hydroxypyruvate reductase. Serine-hydroxymethyl transferase was in the mitochondria. These algae therefore have peroxisomes as in the leaves of higher plants.

Other enzyme systems present in algal microbodies as well as plant peroxisomes, are the glyoxylate cycle and fatty acid β-oxidation. These enzymes have been established in only a few algae (42). Given the limited data, it appears that there are two groups of algal microbodies, unspecialized microbodies containing catalase and uricase, and microbodies similar to leaf peroxisomes of higher plants.

Evolution: Ideas on the evolution of green algae have been developed from cytological and biochemical evidence. Pickett-Heaps (37) and Stewart and Mattox (47) divide the green algae into two basic evolutionary lines. The first line, called the chlamydomonad line, never evolved past the algal stage, and the organisms have the following characteristics: (a) symmetric motile cells; (b) anteriorly attached flagella associated with four crucially arranged, narrow microtubular roots; (c) cell division in which the mitotic spindle disperses after nuclear division with the two daughter nuclei coming close together, another set of microtubules arising perpendicular to the former position of the microtubules of the mitotic spindle, and the new cell wall forming along these microtubules; (d) the presence of glycolate dehydrogenase.

The second evolutionary line eventually led to the formation of the higher plants, and it is characterized by the following: (a) asymmetric motile cells with the flagella attached in a lateral position; (b) flagella roots that consist of a single broad band of microtubules; (c) cell division in which the spindle does not disintegrate but holds the two daughter nuclei apart while the new cell wall is formed by a phragmoplast or by a phragmoplast associated with an infurrowing of the parent plasmalemma; (d) the presence of glycolate oxidase.

The origin of the two lines of evolution is unknown, their ancestor is referred to as an "archetypal unicellular flagellate".

CO_2 Concentration: Some unicellular algae are very efficient in their utilization of CO_2. These algae are CO_2-saturated for photosynthesis at CO_2 concentrations less than atmospheric levels, have a very low CO_2 compensation point, and photosynthesis is not inhibited by O_2 (3,6).

These effects are seen when the algae are grown on low levels of CO_2 (air levels). When grown on high (1-5%) CO_2, the algae have high CO_2 requirements and high CO_2 compensation points.

The ribulose bisphosphate carboxylase-oxygenase of these algae has a high $Km(CO_2)$ and oxygenase activity as in C_3 plants, which do not change when grown on high or low CO_2. Air grown cells can accumulate inorganic carbon (C_i) internally to levels higher than can be accounted for by passive CO_2 diffusion. CO_2-grown cells do not accumulate C_i. Therefore, it has been proposed that air grown cells develop a CO_2 concentrating mechanism that increases CO_2 at the site of RUBISCO.

The species of CO_2 that enters the cell varies. All the blue-green algae tested (4,20,29) and the green algae Scenedesmus (13) take up HCO_3^- from the environment. On the other hand, Chlamydomonas reinhardtii, Chlorella vulgaris, Dunaliella, and other green algae take up CO_2 (33,52,1). Two different mechanisms are proposed depending on whether HCO_3^- or CO_2 enters the cell.

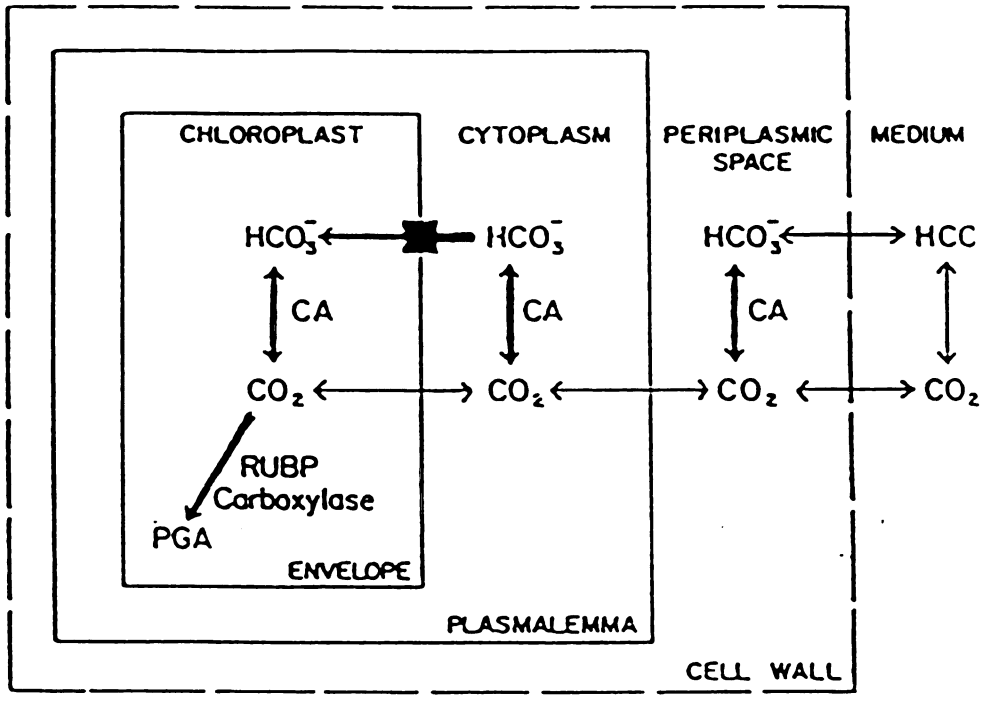
The first mechanism is a carbonic anhydrase facilitated diffusion of CO_2 across the plasma membrane. Some algae with this mechanism have large amounts of carbonic anhydrase (CA) located in their periplasmic space (1,9). CO_2 is apparently the only species of C_i that enters the cell, as determined by short term fixation experiments using either CO_2 or HCO_3^- (53), by the dependence of CO_2 fixation with external pH (33), and by the use of membrane-impermeant inhibitors of CA (32). The membrane-impermeant CA inhibitor, acetazolamide (AZ), severely inhibits CO_2 fixation and C_i accumulation at high external pH, but has little or no effect at low external pH (32). The mechanism may also involve internal carbonic anhydrases. Figure 2 shows a model proposed by

Moroney and Tolbert (32). The external CA replenishes the cell with CO_2 that crosses the plasmalemma, the CO_2 in the cytoplasm then is converted to HCO_3^- by a cytoplasmic CA. The HCO_3^- is then transported into the chloroplast by a HCO_3^- transporter on the chloroplast envelope. A chloroplast CA then replenishes the CO_2 derived from HCO_3^- in the basic chloroplast stroma for RUBISCO. If the internal CA is innibited by the membrane-permeant inhibitor, ethoxzolamide, photosynthesis is reduced even when the external pH is acidic (32). At the same time, though, internal HCO_3^- builds up. This suggests that although inorganic carbon is still accumulating, it is trapped in the chloroplast in a form not available to RUBISCO. This effect is also seen in a mutant that lacks an internal CA, probably because the mutant lacks the chloroplast CA and not the other CA forms (40). These results support a location for the accumulation step at the chloroplast envelope, as CO_2 crosses the plasmalemma yet HCO_3^- is accumulating within the cell.

The second mechanism of CO_2 concentration involves direct uptake of HCO_3^- from the environment. No periplasmic CA is present in these algae and they photosynthesize efficiently even at an external pH of 10 where the concentration of CO_2 in the media is very small. Active transport of HCO_3^- is presumed to be at the plasmalemma and is possibly driven by cyclic electron transport around photosystem I in cyanobacteria (35).

Having a CO_2 concentrating mechanism is an obvious advantage for an algal cell. The algae could grow at CO_2 concentrations much below the CO_2 compensation point of C_3 plants, such as is found in alkaline waters. Concentration of C_i would also reduce photorespiration by increasing the

Figure 2. A Model Of The CO_2 Concentrating Mechanism In Chlamydomonas reinhardtii. From reference 33.


CHLOROPLAST
CYTOPLASM
PERIPLASMIC SPACE
MEDIUM
HCO_3^-
HCO_3^-
HCO_3^-
HCC
CA
CA
CA
CO_2
CO_2
CO_2
CO_2
RUBP Carboxylase
PGA
ENVELOPE
PLASMALEMMA
CELL WALL

carboxylase reaction and reducing the oxygenase reaction of RUBISCO. The carboxylation rate/oxygenation rate was almost 10 times higher for air grown than CO_2-grown C. reinhardtii (31).

PART 1

A Survey of Glycolate Metabolism and CO_2 Concentrating Mechanisms in Green Algae

MATERIALS AND METHODS

Growth Of Algae: The algae used in the study are listed in Table 1 with their growth media. One liter of culture was grown in 3 liter Fernbach flasks under illumination of 150 $\mu E\ m^{-2}\ s^{-1}$ and constant shaking. The cultures were aerated with either air (0.04% CO_2) or air supplemented with 2 to 5% CO_2. Cells from the log phase of growth were harvested by centrifugation at 1000 g for 5 minutes, washed once in distilled water, and repelleted at 10,000 g for 5 minutes. The cells then were resuspended in either 25 mM HEPES at pH 7.3 or the homogenization buffer.

Preparation Of Cell Free Extracts: The algal cells were broken by passing through a Yeda press at 1500 psi N_2. The homogenization buffer for most algae was 50 mM K phosphate at ph 7.0 with 0.01% Triton X-100. Netrium and Mesotaenium were broken in 50 mM tricine at pH 7.5 with 0.01% triton X-100 and 1% PVP. The cell debris was then removed by centrifuging at 500 g for 10 minutes, and the supernatants were used for the assays.

Enzyme Assays: Glycolate dehydrogenase and glycolate oxidase were assayed in Thumberg tubes by following the anaerobic reduction of DCPIP at 600 nm with a Gilford recording spectrophotometer (34). The reaction was done in a 2.5 ml volume containing 0.08 mM Na pyrophosphate at pH 8.5, 0.12 mM DCPIP, 0.1 mM FMN, and either 8 mM glycolate, 20 mM

D-lactate, or 20 mM L-lactate. The sensitivity to cyanide was tested by assaying in the presence of 2 mM KCN. Reactions were initiated by addition of substrate after reading the endogenous rates for 5 minutes. Glycolate dependent O_2 uptake was also measured in an O_2 electrode. The reaction contained 200 μmoles Na pyrophosphate, 20 μmoles glycolate, and 8 μmoles FMN.

Catalase was measured spectrophotometrically by the disappearance of H_2O_2 at 240 nm (28). Protein was measured by the method of Lowry (27).

Glycolate Excretion: Glycolate excretion was measured with harvested cells in buffer at either pH 7.5 (Netrium and Euglena) or pH 6.3 and 8.3 (C. mouewusii). The cells were incubated in 800-1000 μE m^{-2} s^{-1} of light and at a limiting amount of $NaHCO_3$. One ml samples were removed at 0, 15, 30, and 45 minutes, the cells spun out in the microfuge, and the supernatants assayed for glycolate. AOA was added at a concentration of 2 mM. Glycolate was measured by the Calkins method (8).

Photosynthetic Measurements: Photosynthetic O_2 evolution was measured in a Rank Brothers O_2 electrode. The cells were used at a final concentration of 25 µg Chl/ml upon dilution in either 25 mM MES at pH 6.3, 25 mM HEPES at pH 7.3, or 25 mM EPPS at pH 8.3. The buffers were prepared daily and were bubbled with N_2 to lower the dissolved CO_2 and O_2. Three ml of the cell suspension in the O_2 electrode chamber at 25°C were illuminated with 800 µE m^{-2} s^{-1} of light filtered through $CuSO_4$ to remove heat.

Two methods were used to measure the $K_{0.5}(C_i)$ for photosynthesis. The first method was to follow a single progress curve (56) for O_2 evolution after addition of about three times the amount of $NaHCO_3$ needed for half-maximal rates of photosynthesis. The C_i concentration when the O_2 evolution rate was 50% of maximum was then calculated, assuming that the O_2/CO_2 net exchange ratio was one. The second method was to add known amounts of $NaHCO_3$ and measure the initial rate of O_2 evolution for each concentration. This method was used at high pH and for CO_2 grown cells. Before addition of $NaHCO_3$ in both assays, the endogenous CO_2 was depleted by illuminating the algae until O_2 evolution ceased.

The presence of periplasmic CA was determined by the addition of the CA inhibitor acetazolamide (AZ) to photosynthesizing cells. AZ was used at a concentration of 50 mM. CA activity was determined by measuring the time required for the pH to drop from 8.0 to 7.0 in a reaction mixture containing 1.5 ml of 22 mM sodium barbital at pH 8.3, 1 ml of CO_2 saturated water, and 200 µl of cell suspension. All reagents were at 4°C. To measure endogenous rates, AZ was added to inhibit any CA activity.

TABLE 1 Algae, Source, And Growth Media

Algae	Media
Netrium digitus var.digitus UTEX 1257	Watt-Fogg + 5% soil extract (22)
Netrium digitus var.lamellosum UTEX 1256	Watt-Fogg + 5% soil extract
Mesotaenium caldorium UTEX 283	Watt-Fogg + 5% soil extract
Gonium pectorale Muller UTEX LB826	Minimal (49)
Chlorococcum minutum Starr UTEX 1259	Minimal
Ulothrix fimbriata Bold UTEX LB638	Watt-Fogg + 5% soil extract
Chlamydomonas moewusii Duke CC55	Minimal
Euglena gracilis Klebs UTEX LB753	Minimal + vitamins B_1 and B_{12}
Spirogyra varians UTEX LB479	DyIII (24)
Chlorella vulgaris 11h	Minimal
Mougeotia sp UTEX LB758	DyIII
Chlamydomonas reinhardtii 90 UTEX	Minimal

RESULTS

Enzyme Survey: The presence of either glycolate dehydrogenase or glycolate oxidase was determined by the differences in sensitivity to cyanide and D or L-lactate substrate specificity. Glycolate dehydrogenase is inhibited by 2 mM cyanide and oxidizes D-lactate but not L-lactate. Glycolate oxidase is not inhibited by cyanide and oxidizes L-lactate but not D-lactate. DCPIP was used as the electron acceptor in the reaction since the natural acceptor for glycolate dehydrogenase is unknown. Cells grown on air were compared to cells grown on high CO_2 to see whether any change occurred in the type of enzyme.

The results of the enzyme survey are shown in Table 2. One group of the algae studied had glycolate dehydrogenase. Besides glycolate they oxidized D-lactate with activities ranging from 27-68 % that of glycolate. Chlorococcum, however, oxidized D-lactate with a rate 164% higher than glycolate. Specific activities in the cell extracts with glycolate ranged from 2.2-167 nmoles/hr/mg protein. No activity with L-lactate was found for any of these algae. Cyanide inhibited the dehydrogenase activities in these extracts.

Mesotaenium and two species of Netrium contained glycolate oxidase, since 100% of the activity was retained in the presence of 2 mM cyanide and L-lactate was also oxidized. L-lactate rates were 22-83% that of glycolate as previously observed for peroxisomal glycolate oxidase. However, D-lactate was also oxidized with rates 31-58% that of glycolate, which is not observed with enzymes from C_3 plants. Specific activities with glycolate were 1.2-58.0 nmoles/hr/mg protein. Nevertheless, no activity could be found using the glycolate or lactate

TABLE 2 Glycolate and Lactate Oxidation by Algal Extracts

Algae	Glycolate	D-lactate	L-lactate	Catalase	Glycolate	D-lactate	L-lactate
	nmoles/nr/mg protein				% activity with 2 mM CN		
Ulothrix							
air grown	4	2	0	68	0	0	
CO_2 grown	3	2	0	72	0	0	
Gonium							
air grown	2	1.5	0	230	0	0	
Chlorococcum							
air grown	39	64	0	120	0	0	
Chlamydomonas reinhardtii							
air grown	228	137	0		0	0	
Euglena							
air grown	167	59	0	677	5	0	
CO_2 grown	116	40	0	0	10	20	
Chlorella							
air grown	26	7	0	168	17	35	
CO_2 grown	35	10	0	160	25	35	

TABLE 2 Cont.

Algae	Glycolate	D-lactate	L-lactate	Catalase	Glycolate	D-lactate	L-lactate
	nmoles/hr/mg protein				% activity with 2 mM CN		
Netrium 1255							
air grown	20	9	15	8,000	100	100	100
CO_2 grown	14	5	11	6,675	100	100	100
Netrium 1256							
air grown	58	18	13	11,500	100	100	100
CO_2 grown	46	12	11	9,580	100	100	100
Mesotaenium							
air grown	1.2	0.7	1	4,100	100	100	100
CO_2 grown	1.7	0.8	1	3,600	100	100	100
Spirogyra							
air grown	3800	380	2200	16,200	100	100	100

dependent O_2 uptake assay. This lack of detectable activity might be attributed to the less sensitive O_2 uptake assay, although large aliquots of extract were tried from numerous cultures. It is possible that the glycolate oxidizing enzyme in these algae does not link directly to O_2. The algae are still morphologically unicellular, but in solution form clumps or stick together.

No evidence was found for a change in the form of the glycolate oxidizing enzyme when the algae were grown on air or high CO_2. Considerable speculation in the literature has dealt with a higher level of glycolate dehydrogenase in unicellular algae when grown on air relative to high CO_2. However, my results are similar to those previously reported and suggest only a small increase of glycolate dehydrogenase activity in air grown cells. In fact, in Chlorella vulgaris 11h, the enzyme from CO_2 cells was somewhat more active. In view of biological variation, the modest changes in glycolate dehydrogenase between air and CO_2 grown cells is not highly significant.

Catalase was found in all of the algae, but the values varied considerably. Highest catalase activity was found in the algae that had glycolate oxidase, with values of 4.1-11.5 μmoles/hr/mg protein. Algae with glycolate dehydrogenase had catalase activity values of 68-675 nmoles/hr/mg protein. The results are consistent with the development of a more active peroxisomal system in the algae with glycolate oxidase.

CO_2 Concentrating Mechanism: The presence of a CO_2 concentrating mechanism was determined by measuring the $K_{0.5}$ (C_i) at various pH values, and by measuring the presence of external carbonic anhydrase. Whether the cells took up HCO_3^- or CO_2 was determined by the change in $K_{0.5}$ (CO_2) and $K_{0.5}(HCO_3^-)$ over a ph range of 6.3-8.3. If CO_2

entered the cell, the $K_{0.5}(HCO_3^-)$ increased and the $K_{0.5}(CO_2)$ stayed constant with increasing pH. If HCO_3^- entered the cell, the $K_{0.5}(CO_2)$ increased and the $K_{0.5}(HCO_3^-)$ stayed constant with increasing pH.

All of the algae surveyed appeared to take up CO_2. Some of them may have a CO_2 concentrating mechanism, also. In Table 3 is listed the $K_{0.5}(CO_2)$ and external CA activity, if present. C. reinhardtii, C. moewusii, and Gonium definitely have a CO_2 concentrating mechanism, since their $K_{0.5}(CO_2)$ values are very low, 1-2 μM. They differ in that C. reinhardtii and Gonium have external CA, whereas C. moewusii does not. C. moewusii is discussed in detail later in Part 2.

Euglena and Chlorococcum also adapt to growth on air levels of CO_2 by inducing an external CA and a lower $K_{0.5}(CO_2)$. The $K_{0.5}(CO_2)$ of air grown cells is not as low as other algae that have been reported to concentrate CO_2, with values of 6-9 μM instead of 1-3 μM. When grown on high CO_2, no CA was detectable and the $K_{0.5}(CO_2)$ values were 20-30 μM. Euglena has about 2.5 times the level of CA as Clorococcum, and about 1.5 times less than Gonium. Acetazolamide increased the $K_{0.5}(CO_2)$ about 1.5 to 5 fold in the algae with external CA at pH 8.3 as expected (34) (Table 4). No increase in $K_{0.5}(CO_2)$ occurred at pH 5.1 or 6.3, and there was a much lower effect at pH 7.3, indicating that AZ did not enter the cell and inhibit an internal CA.

Netrium and Mesotaenium do not have a CO_2 concentrating mechanism. When grown on air or high CO_2, the $K_{0.5}(CO_2)$ of these algae is 20-35 μM. No external CA was detected by either the CA assay or an AZ effect.

The $K_{0.5}(CO_2)$ values of Spirogyra, Mougeotia, and Ulothrix are intermediate. These algae were more difficult to assay because tney are

TABLE 3 The Presence of a CO_2 Concentration Mechanism In Algae. Assays were run at pH 3.3.

	$K_{0.5}$ (CO_2)		
	air grown	CO_2 grown	Carbonic anhydrase
	μM	μM	units/mg Chl
Ch. reinhardtii	1	25	200
Euglena	7.5	25	79
Gonium	2	24	111
Chlorococcum	7	26	33
Ch. mouewusii	1	9-22	0
Ulothrix	8	10	0
Netrium 1255	35	30	0
Netrium 1256	23	25	0
Mesotaenium	19	23	0
Spirogyra	13	16	0
Mougeotia	16	18	0

TABLE 4 Effect of Acetazolamide On $K_{0.5}$ (CO_2) at pH 8.3 For Air Grown Algae

Algae	- AZ	+ AZ
	μM	μM
Euglena	6.5	26
Chlorococcum	6.9	9.1
Gonium	1.6	9.3
Ch. reinhardtii	1	3.5

filamentuous and their rates of photosynthesis are low. The $K_{0.5}(CO_2)$ values of Spirogyra and Mougeotia were 13-16 μM which are in between the values of air grown unicellular algae with a CO_2 concentrating mechanism and algae without it. No AZ inhibition of CO_2 fixation was noted, and the $K_{0.5}(CO_2)$ was the same when grown on air or CO_2. Ulothrix had a lower $K_{0.5}(CO_2)$ of 8 μm with either air or CO_2 grown cells, and no external CA. Whether these algae have a CO_2 concentrating mechanism cannot be determined on the basis of this data.

Glycolate Excretion: Glycolate excretion was investigated in the unicellular algae Euglena, C. moewusii, C. reinhardtii, and Netrium (Table 5). For Euglena, very little if any glycolate was excreted without AOA in either air grown or CO_2 grown cells. With AOA, the air and CO_2 grown cells excreted a small but equal amount of glycolate. Air grown cells of C. moewusii excreted glycolate at pH 6.3 and 8.3 only if AOA was present. CO_2 grown cells excreted glycolate with or without AOA, but addition of AOA resulted in greater excretion. The CO_2 grown cells excreted 2.5-3 times more glycolate than air grown cells. Netrium did not excrete glycolate in an amount detectable by the Calkins assay, although chromatograms run on ^{14}C-labeled fixation products of the cell showed the presence of glycolate with and without AOA.

TABLE 5 Glycolate Excretion By Selected Algae

Algae	Glycolate excretion
	μmoles/hr/mg Chl
Euglena	
air cells pH 7.5	0.5
air cells + AOA	1.3
CO_2 cells	0.0
CO_2 cells + AOA	1.1
Ch. moewusii	
air cells pH 8.3	0.0
air cells pH 8.3 + AOA	4.2
CO_2 cells pH 8.3	6.7
CO_2 cells pH 8.3 + AOA	10.3
air cells pH 6.3	0.0
air cells pH 6.3 + AOA	3.6
CO_2 cells pH 6.3	6.8
CO_2 cells pH 6.3 + AOA	11.3
Ch. reinhardtii	
air cells pH 7.5	0.1
air cells pH 7.5 + AOA	5.1
CO_2 cells pH 7.5	8.0
CO_2 cells pH 7.5 + AOA	14.8
Netrium air and CO_2 cells	
pH 7.5	0.0

DISCUSSION

The presence of glycolate oxidase or glycolate dehydrogenase in the limited number of algae surveyed did not correlate on the basis of whether the algae were unicellular or multicellular. Most of the algae containing glycolate oxidase that have been examined by others are large, multicellular algae, but some morphologically similar algae have glycolate dehydrogenase. The unicellular algae Netrium and Mesotaenium contained increased catalase and appeared to have glycolate oxidase, yet I was not able to measure O_2 uptake in these extracts.

The type of glycolate oxidizing enzyme is also not dependent on the presence or absence of peroxisomes. From E.M. reports it is clear that most, if not all, green algae have some small microbodies that contain some catalase. Many of these algae also have glycolate dehydrogenase. In general, when glycolate oxidase is present, there is a large increase in catalase and probably an increase in the size and/or number of peroxisomes (44).

The green algae have been divided into two lines of evolution (37,47), those that evolved into higher plants and those that did not. All of the algae with glycolate oxidase are in the algal lines that evolved to higher plants, and, with the exception of Acetabularia and Codium, all of the algae with glycolate dehydrogenase are in the lines that did not evolve into higher plants (Table 6).

Many green algae have photosynthetic CO_2 fixation characteristics of a CO_2 concentrating mechanism, however, the mechanism may be different among them. The algae studied here all used CO_2 as the species of inorganic carbon that enters the cell, but their affinity for

TABLE 6 Type Of Glycolate Oxidation In Algae Orders

Order	Genus-species	Glycolate dehydrogenase	Glycolate oxidase
Dasycladales	Acetabularia	+	-
Siphonocladales	Valonia sp.	+	-
	Dictyosphaeria versluysii	+	-
Codiales	Codium	+	-
	Caulerpa sertularioides	+	-
	Caulerpa verticillata	+	-
	Halimeda cylindracea	+	-
	Halimeda macroloba	+	-
	Halimeda opuntia	+	-
	Udotea argentea	+	-
Zygnematales	Spirogyra varians	-	+
	Mougeotia sp.	-	+
	Netrium digitus	-	+
	Mesotaenium caldorium	-	+
Volvocales	Chlamydomonas reinhardtii	+	-
	Gonium pectorale	+	-
	Chlorogonium elongatum	+	-
	Dunaliella	+	-
Chlorococcales	Chlorococcum (several species)	+	-
Chlorellales	Chlorella vulgaris	+	-
	Eremosphaera	+	-
	Oocystis polymorpha	+	-
Ulotrichales	Microspora sp.	+	-
	Stigeoclonium helveticum	+	-
	Klebsormidium flaccidum	-	+
	Coleochaete scutata	-	+
	Enteromorpha flexuosa	+	-
	Ulothrix fimbriata	+	-
Euglenales	Euglena gracilis	+	-

C_i differed considerably. Air grown Euglena and Chlorococcum have a $K_{0.5}(CO_2)$ of about 7 μM, and Gonium, C. reinhardtii, and C. moewusii have a $K_{0.5}(CO_2)$ of 1-2 μM. External CA is present in Euglena, Chlorococcum, C. reinhardtii and Gonium, but absent in C. moewusii. The importance of the external CA for CO_2 utilization by these algae is questioned when C. moewusii can efficiently utilize CO_2 without it.

All of the algae known to have a CO_2 concentrating mechanism also have glycolate dehydrogenase and not glycolate oxidase. Though this observation might indicate a role for mitochondrial glycolate metabolism in CO_2 concentration, we have not been able to obtain supportive data for this hypothesis. Evidence against this idea was presented by Husic and Tolbert (17) who used a mitochondrial respiration mutant of Chlamydomonas reinhardtii. This mutant lacked cyanide sensitive cytochrome C respiration, but had the cyanide-insensitive respiratory pathway that is inhibited by salicylhydroxamic acid (SHAM). When SHAM was added to the mutant cells, glycolate oxidation was blocked, resulting in accumulation and excretion of glycolate. Oxidation of D-lactate was also blocked. Air grown cells of these mutants had the normal CO_2 concentrating mechanism of wild type cells, since they had a low $K_{0.5}$ (CO_2) (1 μM) and external CA. No effect on the $K_{0.5}(CO_2)$ was seen in the presence of SHAM, thus glycolate and D-lactate oxidation are probably not involved in CO_2 concentration.

No conclusions can be made about the $K_{0.5}(CO_2)$ and CA survey data until more is known about the mechanism of CO_2 concentration. Also, no conclusions can be made about an involvement of glycolate metabolism with CO_2 concentration since the method of CO_2 concentration may be different in the algae although glycolate metabolism may be the same.

The fact remains that no algae with glycolate oxidase has been found that also has a CO_2 concentrating mechanism, even though it would be very beneficial to the algae. If there is an involvement of glycolate metabolism in CO_2 concentration, it probably doesn't involve glycolate oxidation in the mitochondria.

It has been hypothesized that during evolution, algae changed from having glycolate dehydrogenase to glycolate oxidase and at the same time lost their CO_2 concentrating mechanism. From the genetic and morphological evidence presented to date, insignificant substantiative data is available for this hypothesis. Two lines of algae arose, one with glycolate dehydrogenase and a CO_2 concentrating mechanism, and another line with glycolate oxidase and no CO_2 concentrating mechanism, which led to higher plants. In this survey, Chlorella, Euglena, Chlorococcum, Gonium, Ulothrix, and Chlamydomonas with glycolate dehydrogenase belong to the group that did not lead to higher plants, while Spirogyra, and the two unicellular algae, Netrium and Mesotaenium, with glycolate oxidase belong to the group that evolved into higher plants.

PART 2

The CO_2 Concentrating Mechanism of Chlamydomonas moewusii

INTRODUCTION

Many unicellular algae adapt to the low level of CO_2 during growth on air by inducing a CO_2 concentrating mechanism. These algae are saturated for photosynthesis at much lower concentrations of CO_2 and have a very low, O_2 insensitive, CO_2 compensation point (3,6). They are able to concentrate inorganic carbon (C_i) to levels higher than the external concentration (3). The $K_{0.5}(CO_2)$ for photosynthesis is much lower (0.2-2.0 μM) than the $Km(CO_2)$ for ribulose bisphospnate carboxylase isolated from the same algae (25-50 μM CO_2). It is thought that these algae are able to concentrate CO_2 at the site of ribulose bis phosphate carboxylase, thus increasing CO_2 fixation and reducing photorespiration.

The species of C_i that enters the algal cell varies. Any blue-green algae (4,20,29) and the green alga Scenedesmus (13) take up HCO_3^- from the environment. On the other hand, Chlamydomonas reinhardtii (33), Chlorella vulgaris (52), Dunaliella (1), and Euglena (unpublished) take up CO_2 from the media. The ability to utilize CO_2 is often correlated with the induction of a periplasmic carbonic anhydrase (1,9,21). Because carbonic anhydrase catalyzes the equilibrium between HCO_3^- and CO_2, it is more important to the algae at high pH where the amount of CO_2 is limiting. Periplasmic carbonic anhydrase has not been reported in algae that take up HCO_3^-.

Except for the periplasmic carbonic anhydrase (CA), little is known about the CO_2 concentrating mechanism. A scheme postulated by Moroney,

et al (33) for C. reinhardtii requires the following proteins: periplasmic CA to replenish CO_2 from HCO_3^- as CO_2 enters the cell; a cytoplasmic CA to convert the CO_2 to HCO_3^-; a HCO_3^- transporter on the chloroplast envelope to transport HCO_3^- into the chloroplast; and a chloroplast CA to supply CO_2 for ribulose bisphosphate carboxylase. The location of the internal carbonic anhydrases and the HCO_3^- transporter has not been established, although recent work (Moroney et al unpublished) with isolated intact C. reinhardtii chloroplasts supports the contention that a HCO_3^- transporter is located on the chloroplast envelope.

The photosynthetic properties of another Chlamydomonas species, C. mouewusii, are described here. Unlike C. reinhardtii, this organism does not have a periplasmic carbonic anhydrase, although other characteristics of its CO_2 concentrating mechanism are similar. By comparing the two species the physiological significance of the periplasmic carbonic anhydrase can be discussed.

MATERIALS AND METHODS

Chlamydomonas moewusii, strain CC55, from the algae collection at Duke University, Chlamydomonas reinhardtii, strain 90, and Chlamydomonas reinhardtii, strain CW 15, from the algae collection at the University of Texas-Austin were grown phototrophically on a minimal medium (49). One liter of culture was grown in 3 liter Fernbach flasks under illumination of 100 $\mu E/m^2/s$ and constant shaking. The cultures were aerated with either air or air supplemented with 3-5% CO_2 and cells from the log phase of growth were harvested by centrifugation at 1000 g for 5 minutes. The cells were washed once in H_2O and repelleted at 10,000 g for 5 minutes. They were then resuspended in a few ml of 25 mM HEPES-KOH at pH 7.3 and stored on ice.

Photosynthetic O_2 evolution was measured in a Rank Brothers O_2 electrode. The cells were used at a final concentration of 25 µg Chl/ml upon dilution in the buffers listed in Table 7. The buffers were prepared daily and bubbled with N_2 to lower the dissolved CO_2 and O_2. Three ml of the cell suspension in the O_2 electrode chamber at 25°C were illuminated with 800 $\mu E/m^2/s$ of light filtered through $CuSO_4$.

Two methods were used to measure the $K_{0.5}(C_i)$ for photosynthesis. The first method was to follow a single progress curve (56) for O_2 evolution after addition of about three times the amount of $NaHCO_3$ needed for half-maximal rates of photosynthesis. The C_i concentration when the O_2 evolution rate was 50% of maximum was then calculated assuming that the O_2/CO_2 net exchange ratio was one. The second method was to add known amounts of $NaHCO_3$ and calculate the initial rate of O_2 evolution for each concentration. This method was used at high pH and for CO_2-grown cells. Before addition of $NaHCO_3$ in both assays, the endogenous CO_2 was depleted by illuminating the algae until O_2 evolution stopped.

The accumulation of C_i was measured by the silicone oil filtration technique (3,16,22). Assays were done in the light at 400 $\mu E/m^2/s$ in 400 µl microfuge tubes in a Beckman microfuge. The tubes contained (from bottom to top): 25 µl of glycine at pH 10.0 with 0.75% SDS, 65 µl of silicone oil (1:1 of Wacker AR 20 and AR 200), and 250 µl of cells at a concentration of 25 µg Chl/ml previously illuminated to deplete the endogenous CO_2. The reaction was started by adding 30 µM $NaH^{14}CO_3$. After either 15, 30, 45, or 60 s the reaction was terminated by spinning the cells through the oil into the glycine/SDS solution. Internal C_i was measured from the difference between the alkaline and acid stable ^{14}C in the pellet. The intracellular volume was estimated using ^{14}C-sorbital and ^3H_2O (16).

The presence of CA in the algae was determined by the effect of CA inhibitors on photosynthetic O_2 evolution (32) and by an enzyme assay for CA. Acetazolamide (AZ) is a membrane-impermeant inhibitor of CA used to determine the presence of periplasmic CA. Ethoxzolamide (EZ) is a membrane-permeant CA inhibitor used to detect the presence of internal CA. AZ was used at 50 µM and EZ at 100 µM. CA activity was determined by measuring the time required for the pH to drop from 8.0 to 7.0 in a reaction mixture containing 1.5 ml of 22 mM sodium barbital at pH 8.3, 1 ml of CO_2 saturated H_2O, and 200 µl of cell suspension. All reagents were at 4°C. Periplasmic CA was measured with whole cells, and internal CA was measured in broken cells. As controls to measure endogenous rates, AZ or EZ was used to block cellular CA activity.

Proteinase Treatment: A 3 pcv suspension of air-grown cells was incubated in culture medium containing either 0.05% trypsin (w/v) or 3% (w/v) subtilisin for 10, 30, and 90 minutes. The cells were then harvested and washed 3 times at 10,000 g for 5 minutes.

Chlorophyll was measured by dissolving the cells in methanol and reading the absorbance at 650 and 665 nm.

RESULTS

Photosynthetic rates as O_2 evolution at pH 8.3 with and without AZ by air-grown Chlamydomonas reinhardtii and moewusii are compared in Figure 3. The rates of photosynthesis by the two algae were very similar, and AZ had little effect on this rate by C. moewusii. AZ inhibition of the periplasmic CA greatly reduced photosynthesis by C. reinhardtii, unless extremely high concentrations of CO_2 were available for diffusion into the cell.

The species of C_i entering the cells of Chlamydomonas moewusii was studied by measuring the $K_{0.5}(C_i)$ at different pH values (Table 7) (33). The proportion of HCO_3^- and CO_2 in the external medium was varied with pH and determined by the Henderson-Hasselbach equation. The apparent affinity for total inorganic carbon and the $K_{0.5}(HCO_3^-)$ increased logarithmically with increasing pH, but the $K_{0.5}(CO_2)$ remained constant at a low value of about 1 μM. This implies that CO_2 is the predominant C_i species that enters the C. moewusii cells. The low $K_{0.5}(CO_2)$ is close to that for C. reinhardtii, which had a $K_{0.5}(CO_2)$ of 0.80-1.2 μM between pH 6.3 and 9.3 (33, data not shown). The membrane-impermeant CA inhibitors, acetazolamide or a dextran-bound sulfonamide, nad no effect on the $K_{0.5}(CO_2)$ of C. moewusii at pH 8.3 or 9.3. In contrast, the $K_{0.5}(CO_2)$ of C. reinhardtii increased over 5 fold when the periplasmic CA was inhibited by AZ or a dextran-bound sulfonimide at pH 8.3 (32). In addition, no external CA activity was detected with whole cells by the CA assay. These results show that C. moewusii, unlike C. reinnardtii, has no periplasmic CA.

Figure 3. The Rate Of Photosynthetic O_2 Evolution At Increasing External CO_2 Concentration By Air-Grown C. moewusii And C. reinhardtii At pH 8.3. The membrane-impermeant CA inhibitor, acetazolamide, was added just prior to the start of each assay. The cells were illuminated with 800 $\mu E/m^{-2}/s^{-1}$ at a concentration of 25 μg Chl ml^{-1} in 25 mM EPPS at pH 8.3. C. moewusii (o), C. moewusii + AZ (●), C. reinhardtii (△), C. reinhardtii + AZ (▲).

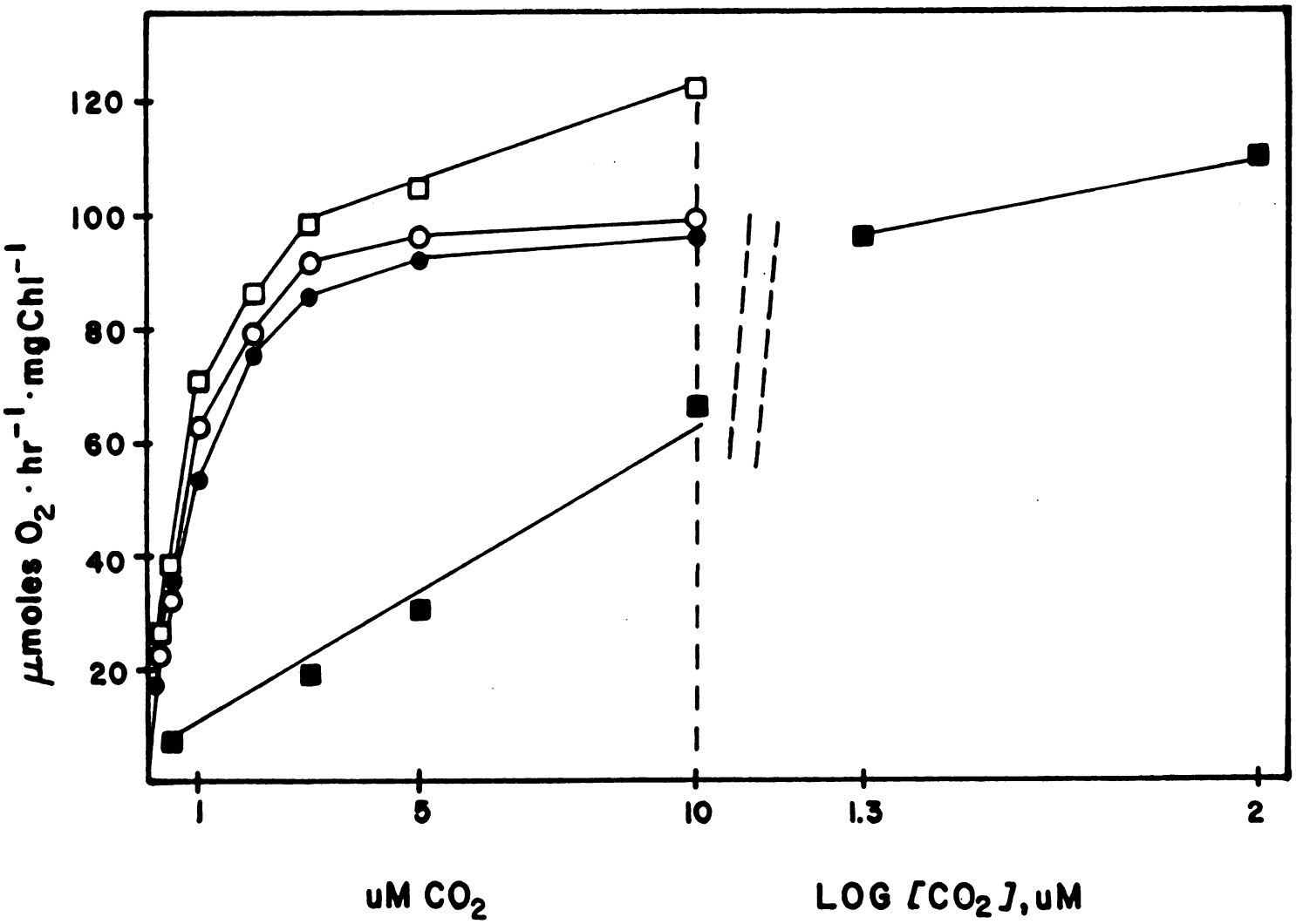

120
100
80
60
40
20
μmoles $O_2 \cdot hr^{-1} \cdot mgChl^{-1}$
1
5
10
1.3
2
uM CO_2
LOG [CO_2], uM

TABLE 7 Inorganic Carbon Requirement For Photosynthetic O_2 Evolution By Air-Grown C. moewusii; The Absence Of An Effect From Acetazolamide (AZ). The cell concentration was 25 μg Chl·ml^{-1} and AZ was 50 μM.

Buffers	pH	$K_{0.5}(C_i)$	$K_{0.5}(HCO_3^-)$	$K_{0.5}(CO_2)$
		μM	μM	μM
25 mM MES	6.3	2	1	1
25 mM HEPES	7.3	9	9	0.9
25 mM EPPS	8.3	91	90	0.9
25 mM EPPS + AZ	8.3	95	94	0.9
25 mM CHES	9.3	962	961	0.9
25 mM CHES + AZ	9.3	1120	1119	1.1

The membrane-permeant CA inhibitor, EZ, raised the $K_{0.5}(CO_2)$ over 10 fold in air grown C. moewusii (Table 8). The increase was 60 fold in C. reinhardtii, which was more affected probably because the external CA would also be inhibited by EZ. Because of the similar EZ effect, an internal CA is probably present in both of these algae. A specific activity of 65 units/mg Chl was detected by the CA assay with broken cells of C. moewusii. This is at least three times the amount of internal CA in the C. reinhardtii wall-less mutant CW 15.

Periplasmic CA was released from the cells of C. reinhardtii when treated with trypsin (54). Trypsin was the most effective proteinase of several tested. Also, the external CA activity of Dunaliella was destroyed in the presence of subtilisn (2). The subtilisn-treated cells lost the ability to use HCO_3^- for photosynthesis at high pH due to the loss of CA activity (1). To see if an external enzyme, CA or other enzyme, was involved in CO_2 concentration by C. moewusii, the effect of trypsin and subtilisn on the $K_{0.5}(CO_2)$ at pH 8.3 was determined. After treatment with the proteinases for up to 90 minutes, no change in the $K_{0.5}(CO_2)$ or V_{max} for photosynthesis was seen. When C. reinhardtii was treated in the same way, the $K_{0.5}(CO_2)$ increased 2.5-3.0 times the value of untreated cells (Table 9). Acetazolamide further increased the $K_{0.5}$ (CO_2) of the trypsin-treated C. reinhardtii cells about 2-fold, showing that some periplasmic CA remained although no CA activity was detectable by the CA assay in whole cells.

An antibody to the periplasmic CA of C. reinhardtii has been prepared (Husic,unpublished). This antibody cross-reacts only weakly with internal CA of C. reinhardtii, with weak bands at 26, 28, 45, 90, and

TABLE 8 Internal Carbonic Anhydrase Activity And The Effect Of Ethoxzolamide On The $K_{0.5}(CO_2)$ Of Air-Grown C. moewusii And Wild-Type C. reinhardtii At pH 7.3. Cell concentration was 25 µg Chl·ml^{-1} in 25 mM HEPES. EZ was 100 µM. The level of internal CA was measured with broken cells. Wall-less C. reinhardtii cells were used to measure internal CA because of the abundant periplasmic CA of wild type C. reinhardtii. The wall-less cells were washed 3 times to remove all external CA before lysis (H. David Husic unpublished).

	$K_{0.5}(CO_2)$		
	Control	+ EZ	Internal CA activity
	µM	µM	units·mg Chl^{-1}
C. reinhardtii	1.0	60	20
C. moewusii	1.1	17	67

TABLE 9 Effect Of Proteinase Treatment On The $K_{0.5}$ (CO_2) Of *C. reinhardtii* At pH 8.3.

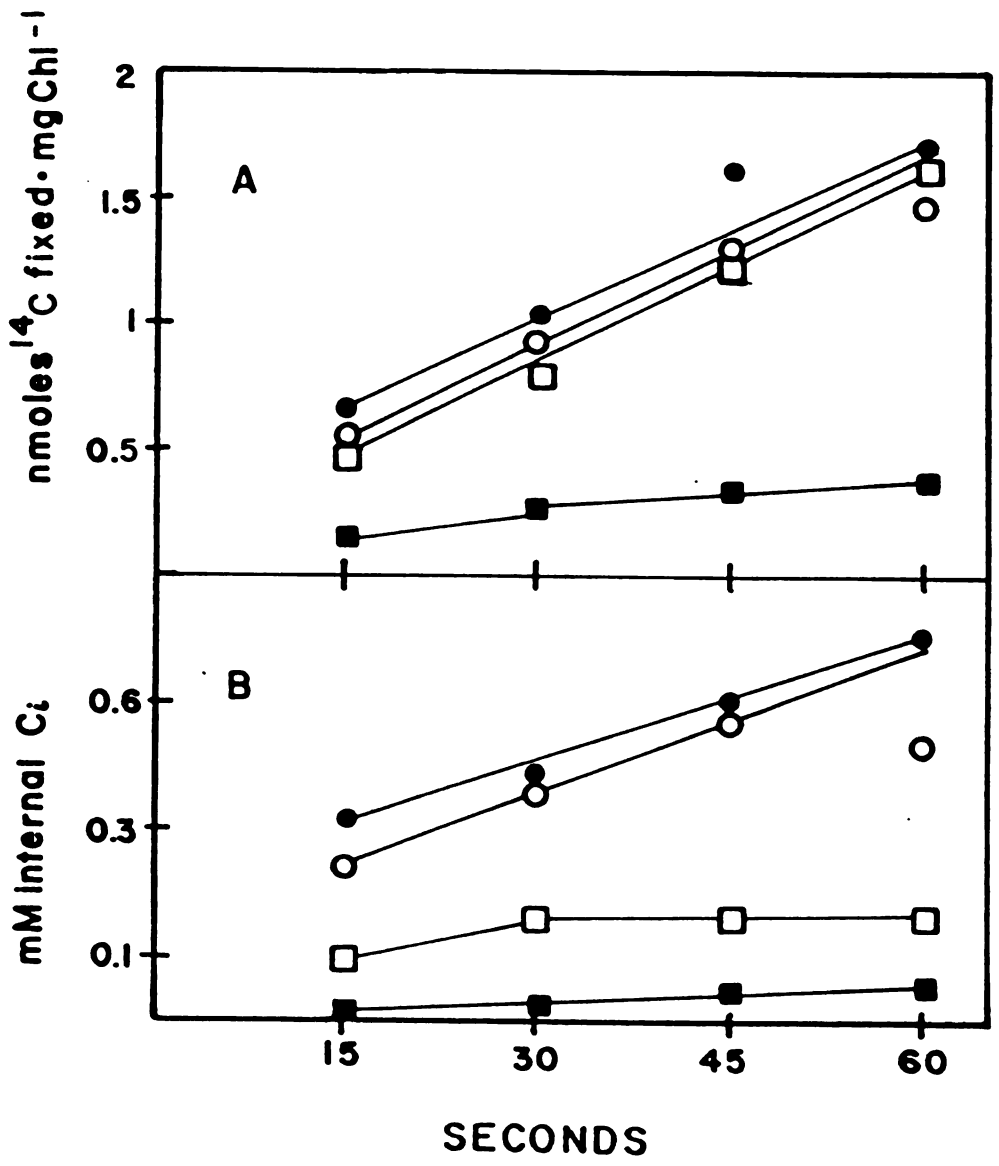
Treatment	$K_{0.5}$ (CO_2)
	μM
Untreated	132
Subtilisn	320
Trypsin	390
Trypsin + AZ	705

130 kDaltons. When a C. mouewusii broken cell extract was probed with the antibody, two strong bands occured at 20 and 22.5 kDaltons with a weaker band at 28 kDaltons (Husic,personal communication). No background reaction was observed. These proteins may be the internal CA of C. moewusii, although they must be different than the internal CA of C. reinnardtii since the molecular weights and degree of cross-reactivity are different. These bands may also represent proteins on the cell surface, though no evidence exists for the actual location.

C. moewusii accumulated C_i internally to at least 10 times the external concentration. Results for experiments at pH 8.3 are presented in Figure 4. Results at pH 7.3 were similar except the rate of CO_2 fixation and amount of C_i were greater due to a higher level of CO_2 at the lower pH from the same amount of added $NaHCO_3$. AZ had no effect on ^{14}C fixation or C_i accumulation by C. moewusii at pH 8.3 or 7.3, but inhibited both in C. reinhardtii. The results are consistent with a lack of periplasmic CA in C. moewusii, yet both algae still accumulate C_i internally. In fact, C. moewusii accumulates C_i to a higher level than C. reinhardtii.

The addition of bovine carbonic anhydrase to photosynthesizing air grown C. moewusii had no effect on the $K_{0.5}(CO_2)$. When 1 mg CA/ml was added to the algal suspension in the O_2 electrode chamber, no enhancement or inhibition of $K_{0.5}(CO_2)$ or V_{max} for photosynthesis was seen at pH 8.3 or 9.3. When external CA was removed by subtilisn treatment from Dunaliella, which needs the CA to utilize HCO_3^- at high pH, photosynthesis was reduced. Added CA restored the photosynthetic rates to normal (1).

Figure 4. $^{14}CO_2$ Fixation And C_i Accumulation By Air-Grown C. moewusii and C. reinhardtii At pH 8.3 And The Effect Of Acetazolzmide. Fixed $^{14}CO_2$ (A) and internal C_i (B) were determined following the addition of 30 μM $NaH^{14}CO_3$. The concentration of cells was 25 μg Chl ml^{-1} in 25 mM EPPS at pH 8.3. AZ concentration was 50 μM. C. moewusii (o), C. moewusii + AZ (●), C. reinhardtii (□), C. reinnardtii + AZ (■).


nmoles ^{14}C fixed · mg Chl $^{-1}$
2
1.5
1
0.5
A
B
mM Internal C_i
0.6
0.3
0.1
15
30
45
60
SECONDS

High CO_2 grown C. moewusii and C. reinhardtii, which should lack the CO_2 concentrating mechanism, were also compared for CO_2 fixation and C_i accumulation. With C. moewusii the $K_{0.5}(CO_2)$ for photosynthesis varied somewhat depending on the external pH (Table 10). However, the $K_{0.5}(CO_2)$ values were 10 times greater than the $K_{0.5}(CO_2)$ of air-grown cells. It appears that C. moewusii undergoes an adaption to low and high CO_2, although an external CA is not involved. The $K_{0.5}(CO_2)$ values at pH 8.3 and 6.3 are 8.5-9.5 μM, which are 2.5 times lower than the 23 μM $K_{0.5}(CO_2)$ value at pH 7.3. Moroney and Tolbert (33) had reported a constant high $K_{0.5}(CO_2)$ for CO_2-grown C. reinhardtii of 22 μM CO_2 independent of pH. When checked again, however, they found the same variation with pH with C. reinhardtii (unpublished) as seen here with C. moewusii.

At pH 7.3 CO_2-grown C. moewusii accumulate C_i almost to the level of air-grown cells (Figure 5B), and much higher than CO_2-grown C. reinhardtii. Nevertheless, the rate of ^{14}C fixation (Figure 5A) was lower for CO_2-grown cells than air-grown cells. At pH 8.3, tne CO_2-grown C. moewusii fixed and accumulated little C_i (Figure 6), although the $K_{0.5}(CO_2)$ at 8-10 μM was intermediate between the $K_{0.5}(CO_2)$ for air-grown and CO_2-grown C. reinhardtii.

TABLE 10 Inorganic Carbon Requirement For 5% CO_2-Grown C. moewusii. Cell concentration was 25 $\mu g \cdot Chl \cdot ml^{-1}$. Buffers used are the same as in Table I.

pH	$K_{0.5}(C_i)$	$K_{0.5}(HCO_3^-)$	$K_{0.5}(CO_2)$
	μM	μM	μM
6.3	19	9	9
7.3	220	198	22
8.3	850	341	9

Figure 5. $^{14}CO_2$ Fixation And Inorganic Carbon Accumulation By High CO_2-Grown C. moewusii (o) And C. reinhardtii (□) At pH 7.3. Experimental conditions are the same as in Figure 4. The buffer was 25 mM HEPES at pH 7.3.

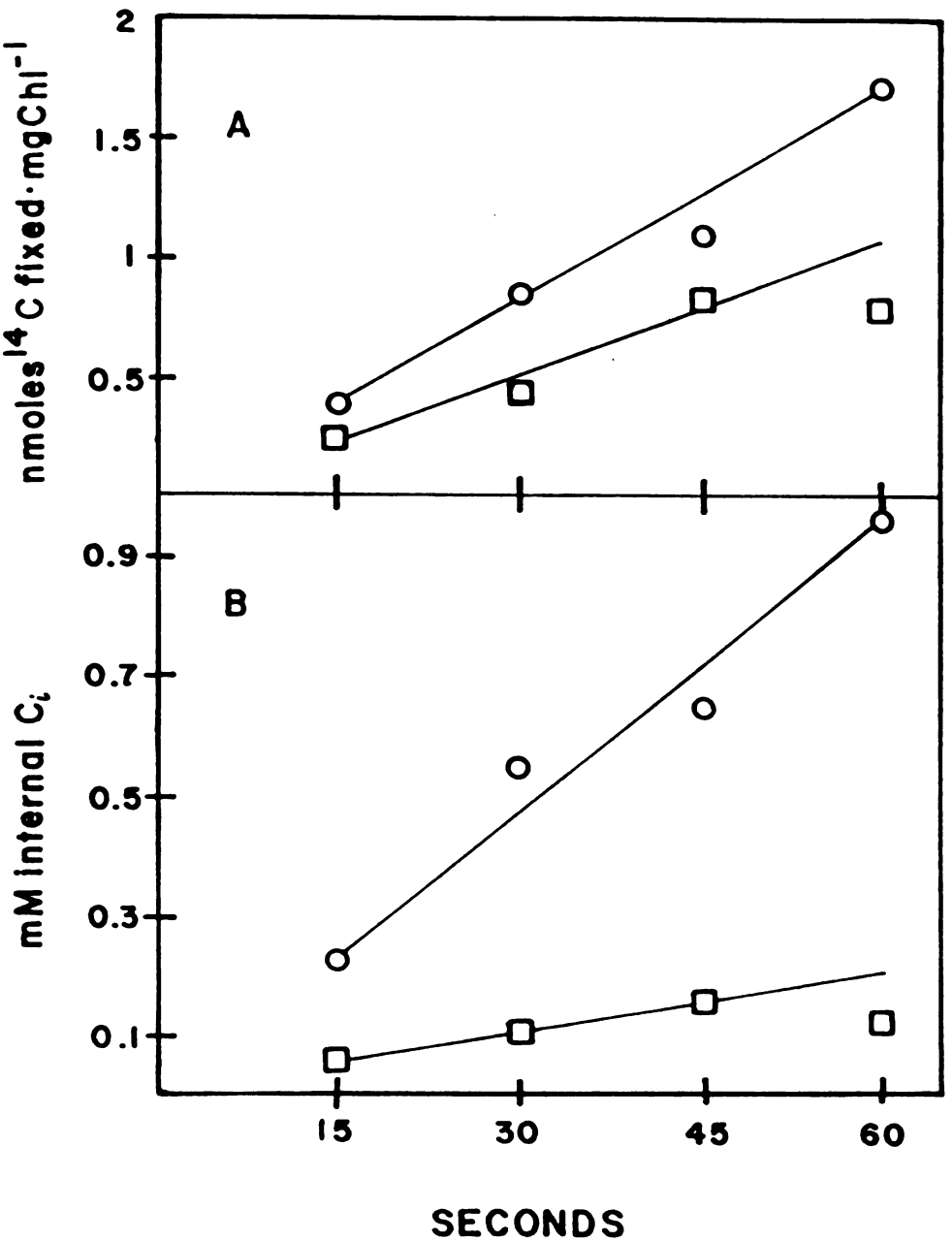
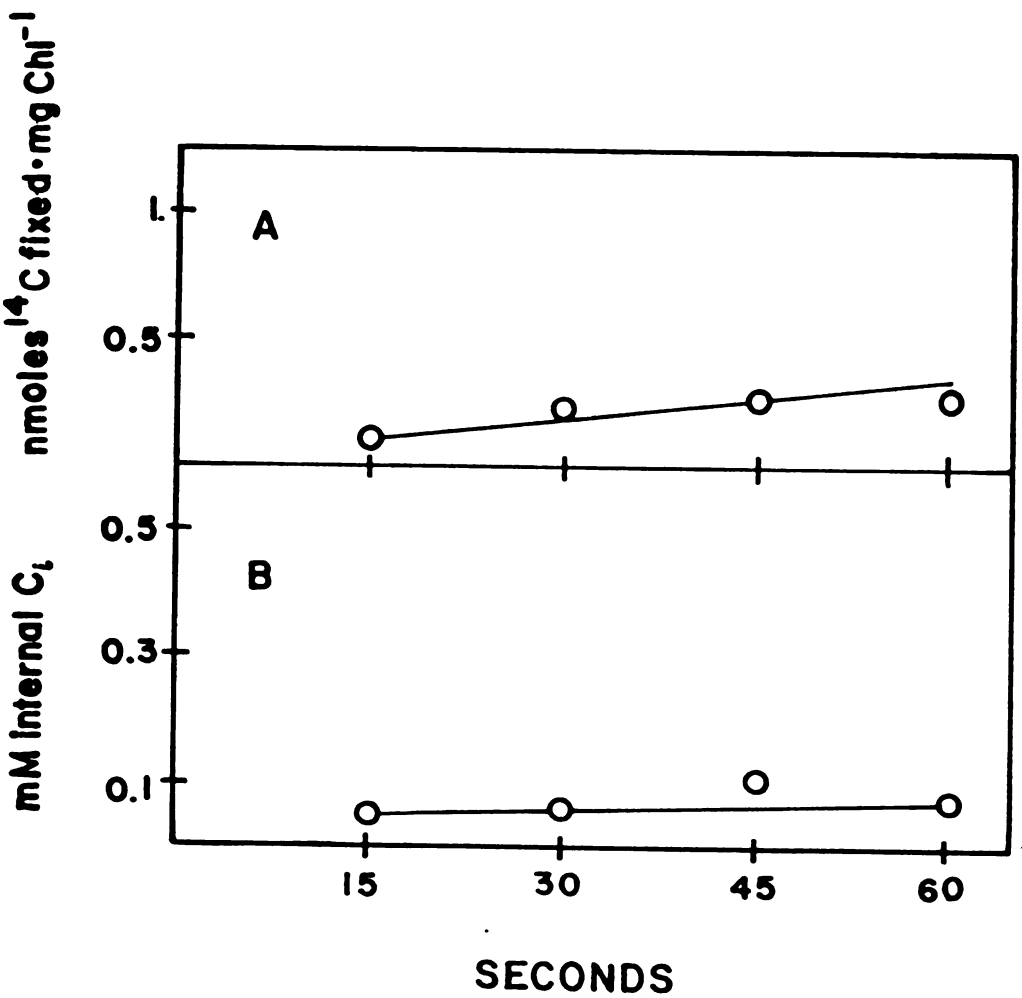

2
1.5
1
0.5
A
nmoles ^{14}C fixed·mgChl^{-1}
0.9
0.7
0.5
0.3
0.1
B
mM internal C_i
15
30
45
60
SECONDS

Figure 6. $^{14}CO_2$ Fixation And C_i Accumulation By High CO_2-grown C. moewusii At pH 8.3. Experimental conditions are the same as in Figure 4.


nmoles ^{14}C fixed · mg Chl^{-1}
mM internal C_i
A
B
1.
0.5
0.5
0.3
0.1
15
30
45
60
SECONDS

DISCUSSION

Periplasmic carbonic anhydrase is an important adaptive part of the CO_2 concentrating mechanism of C. reinhardtii. It is thought that the CA facilitates tne entry of CO_2 from the HCO_3^- pool. When the cells are treated with a membrane-impermeant CA inhibitor, AZ, the $K_{0.5}(CO_2)$ increases about 5 fold and internal C_i accumulation decreases when the pH is over 7.0. Thus, periplasmic CA is important but not solely responsible for efficient CO_2 concentration by C. reinhardtii at high pH where HCO_3^- predominates in solution.

The results presented here show that C. moewusii concentrates C_i with about the same efficiency as C. reinhardtii and apparently takes up CO_2 while lacking the periplasmic CA. If HCO_3^- were entering the cell, the $K_{0.5}(HCO_3^-)$ would be expected to remain constant with increasing pH, and the apparent $K_{0.5}(CO_2)$ would also decrease from CO_2 conversion to HCO_3^-. However, the $K_{0.5}(CO_2)$ remained low and constant while the apparent $K_{0.5}(HCO_3^-)$ increased, as if CO_2 enters the cell. Z had no effect on $K_{0.5}(CO_2)$ or intenal C_i accumulation by C. moewusii, and no external CA activity was detected by the CA assay with whole cells. All the results are consistent with the uptake of CO_2 and lack of periplasmic CA in C. moewusii.

The possibility exists that a small amount of HCO_3^- could be taken up by C. moewusii. Using the publisned values for the hydration/dehydration of HCO_3^- at different pH (12), a computer program was written to determine the time course of the nonenzymatic HCO_3^--CO^2 conversion at various pH values. This was used to determine if the amount of CO_2 present at the various C_i concentrations and pH was enough

to account for the observed rates of photosynthesis. Even at pH 9.3 the amount of CO_2 from the nonenzymatic dehydration of HCO_3^- seemed sufficient to account for the low initial rates of photosynthesis measured with C. moewusii and C. reinhardtii.

In many aspects the CO_2 concentrating mechanism of C. moewusii compares well with C. reinhardtii. The $K_{0.5}(CO_2)$ is basically the same for the two algae as well as the level of internal C_i accumulation. The rate of CO_2-dependent photosynthetic O_2 evolution at increasing CO_2 concentration at nigh pH were very similar (Figure 3). However, AZ severely inhibited photosynthesis by C. reinhardtii at high pH, while it had no effect on C. moewusii.

Despite being of the same genus, C. reinhardtii and C. moewusii are not closely related, on the basis of chloroplast DNA (25). Likewise, the presence or absence of periplasmic CA in these two Chlamydomonas indicate that they are different in their CO_2 concentrating mechanism. A similar difference between various Chlorella species has been reported (30). In some respects, the photosynthetic properties of C. moewusii are similar to a C. reinnardtii mutant without the external CA.

The results from the CO_2-grown cells show that C. moewusii adapts to the level of CO_2 during growtn as does C. reinhardtii. The $K_{0.5}(CO_2)$ was at least 10 times higher than that for air-grown cells. The internal C_i accumulation at pH 7.3 even when the $K_{0.5}(CO_2)$ was 20-25 μM can be explained by assuming a high internal pH. The variability of $K_{0.5}$ (CO_2) with pH can be explained if tne rate limiting step for photosynthesis is the HCO_3^--CO_2 conversion, which is faster at pH 8.3 and 6.3 than at pH 7.3 (12,23). Thus, CO_2-grown cells may lack the CO_2 concentrating mechanism, but if the internal pH is greater than 7.3, the cells could still accumulate C_i.

These results raise the question of why a periplasmic CA is needed in some algae. The amount of CO_2 available from the nonenzymatic dehydration of HCO_3^- appears to be enough to allow CO_2 concentration by C. moewusii to be as efficient as C. reinhardtii. Possibly the other components of the CO_2 concentrating mechanism are more efficient in C. moewusii. Evidence for this is the 3-fold higher level of internal CA in C. moewusii than C. reinhardtii. This may help prevent the loss of CO_2 from back diffusion out of the cell by trapping the CO_2 as HCO_3^-.

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