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Internal Inorganic Carbon Pool of *Chlamydomonas reinhardtii*

EVIDENCE FOR A CARBON DIOXIDE-CONCENTRATING MECHANISM[1]

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ABSTRACT

The external inorganic carbon pool (CO_2 + HCO_3^-) was measured in both high and low CO_2-grown cells of *Chlamydomonas reinhardtii*, using a silicone oil layer centrifugal filtering technique. The average internal pH values were measured for each cell type using [^{14}C]dimethyloxazolidinedione, and the internal inorganic carbon pools were recalculated on a free CO_2 basis. These measurements indicated that low CO_2-grown cells were able to concentrate CO_2 up to 40-fold in relation to the external medium. Low and high CO_2-grown cells differed in their photosynthetic affinity for external CO_2. These differences could be most readily explained as being due to the relative CO_2-concentrating capacity of each cell type. This physiological adaptation appeared to be based on changes in the abilities of the cells actively to accumulate inorganic carbon using an energy-dependent transport system.

The energy dependence of CO_2 accumulation was investigated, using the inhibitors methyl viologen, 3-(3,4-dichlorophenyl)-1,1 dimethylurea, carbonyl cyanide trifluoromethoxyphenylhydrazone, and 3,5-di-*tert*-butyl-4-hydroxybenzylide nemalononitrile. It appears that the concentrating mechanism in both cell types may be dependent upon an energy supply linked to both phosphorylation in general and photophosphorylation. The treatment of low CO_2-grown cells with the carbonic anhydrase inhibitor ethoxyzolamide decreased the apparent photosynthetic affinity for CO_2. This was correlated with a decrease in the transport of inorganic carbon into the cells.

The nature of the CO_2-concentrating mechanism, particularly with respect to a bicarbonate transport system, is discussed, and its possible occurrence in other algae is assessed.

Investigations by Berry *et al.* (2) have indicated that the photosynthetic exchange kinetics (as measured by O_2 evolution) of *Chlamydomonas reinhardtii* are dramatically affected by the previous growth conditions of the algae. Cells grown with air levels of CO_2 (330 μl/l) have a much higher affinity for CO_2 in photosynthesis than cells grown with 50 ml/l CO_2 in air. The increase in photosynthetic affinity for CO_2 is correlated with a reduction in the O_2 sensitivity of photosynthesis as measured by growth, glycolate excretion, and inhibition of net photosynthesis. Both low and high CO_2-grown cells excrete glycolate when exposed to external O_2; however, this is only detected over ranges of external [CO_2] which limit the rate of photosynthesis.

The high affinity of photosynthesis for CO_2 in low CO_2-grown cells ($K_m(CO_2)$ <1 μM at pH 7.0; ref. 2) indicates that *C. reinhardtii* can adapt to become superior in CO_2 utilization to C_3 species of higher plants. Such adaptation has also been shown in *Scenedesmus obliquus* (4) and *Chlorella vulgaris* (10). Evidence presented by Berry *et al.* (2) indicates that the $K_m(CO_2)$ of RuP_2[5] carboxylase is similar in low and high CO_2-grown cells and C_3 plants. In contrast to C_4 plants, no evidence for an alternative initial carboxylation mechanism in *C. reinhardtii* was obtained either from studies of P-enolpyruvate carboxylase activity or the initial products of CO_2 fixation. This is also supported by investigations of high and low CO_2-grown cells of *Chlorella pyrenoidosa* (20). Carbonic anhydrase activity in low CO_2-grown algae has been found to be manyfold higher than in high CO_2-grown cells (6, 7, 17). Furthermore, Graham *et al.* (7) showed that, when high-CO_2-grown cells of *Chlorella* or *Chlamydomonas* were transferred to low CO_2, both carbonic anhydrase and O_2 evolution increased during the first 90 min, without detectable changes in the activity of the reductive pentose phosphate pathway or β-carboxylation enzymes. It has been suggested that these changes in carbonic anhydrase may explain the improved efficiency of low CO_2-grown cells. However, it is questionable whether an increase in carbonic anhydrase activity is in itself sufficient to explain the higher affinity for CO_2 of low CO_2-adapted cells. This enzyme will promote equilibration of CO_2 and HCO_3^-, but the affinity of the system for CO_2, in the presence of carbonic anhydrase, should not exceed that of the carboxylation mechanism.

Studies of the response of $K_m(CO_2)$ and $K_m(HCO_3^-)$ of low and high CO_2-grown *C. reinhardtii* to external pH (2) have shown that high-CO_2-grown cells have a fairly constant $K_m(CO_2)$ from pH 6.5 to 8.0. However, in low CO_2-grown cells, the $K_m(CO_2)$ increased and the $K_m(HCO_3^-)$ decreased over the same pH range. This suggests that low CO_2-grown cells are capable of taking varying proportions of CO_2 and HCO_3^-, depending on the external pH, whereas high CO_2 may use only free CO_2 as the photosynthetic substrate. Berry *et al.* (2) suggested, therefore, that low CO_2-grown cells may actively accumulate HCO_3^- by a metabolic influx pump. This would be the basis of a CO_2-concentrating mechanism and may explain the high affinity for CO_2 of low CO_2-grown cells.

These studies were initiated to test the above hypothesis. The internal CO_2 pool in both high and low CO_2-grown cells as a function of the external CO_2 concentration was determined. The data provide evidence showing that the increased CO_2 affinity of low CO_2-grown cells is the result of a CO_2-concentrating mechanism. The energy dependence of the mechanism was also investigated.

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[5] Abbreviations: Ru-P_2: ribulose 1,5-bisphosphate; SF 6847: 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; FCCP: carbonyl cyanide trifluoromethoxyphenylhydrazone; DMO: 5,5-dimethyloxazolidine-2,4-dione; SIS: sorbitol-impermeable space.

MATERIALS AND METHODS

Growth of Algae. *C. reinhardtii* (haploid strain 137 C, Stock GB-126) was grown phototrophically at 25 C, as described by Berry *et al.* (2). Cultures were bubbled with either air (330 μl/l CO_2) or 50 ml/l CO_2 in air. These two growth conditions were used to obtain low CO_2- and high CO_2-grown cells, respectively.

Radioactive Compounds. Radioactive labeled compounds were purchased from the following suppliers: [U-^{14}C]sorbitol (New England Nuclear, 213 mCi/mmol); ^3H_2O (New England Nuclear; 1 mCi/ml); [2-^{14}C]5,5-dimethyloxazolidine-2,4-dione (New England Nuclear, 46.6 mCi/mmol), $NaH^{14}CO_3$ (Amersham/Searle, 8–10 mCi/mmol).

Preparation of Cells. The cell culture was centrifuged at 2,000*g* for 5 min. The isolated pellet was resuspended in 50 mM Hepes (pH 7.15). (The final cell concentration was generally between 1–2 × 10^8 cells/ml.) This cell suspension was stored on ice, in the dark, for the duration of the experiment. This treatment did not alter the photosynthetic performance of the cells. Prior to incubation of the cells with $NaH^{14}CO_3$, an aliquot of concentrated cell suspension was diluted into degassed suspension buffer in an illuminated O_2-electrode chamber (Rank Bros., 40 nE cm^{-2} s^{-1}, 400–700 nm), with a final concentration of approximately 1.5 × 10^7 cells/ml. The chamber was closed and the cells were allowed to deplete the medium of endogenous CO_2 (measured by the cessation of O_2 evolution). The depletion period was approximately 3 to 5 min for low CO_2 cells and 10 to 15 min for high CO_2 cells. This procedure was necessary to ensure that the low concentrations of $NaH^{14}CO_3$, used in the following incubation, were not diluted by unknown amounts of unlabeled CO_2.

Incubation and Filtering Centrifugation. The incubations were carried out in 400-μl plastic microfuge tubes, in the light (40 nE cm^{-2} s^{-1}, 400–700 nm, at 25 C). The tubes (from bottom to top) contained 20 μl of 1 M glycine (pH 10.0), 0.75% (w/v) SDS, 65 μl silicone oil (1:4 (v/v), SF 96 (50), Versilube F50 (from General Electric, Silicone Products Division), and 300 μl cell suspension which had been pretreated in the O_2-electrode chamber. Incubations were started by the addition of $NaH^{14}CO_3$ and terminated by filtering centrifugation (13) in a Beckman Microfuge B. This was followed by injection of 15 μl of 3 M NaOH into the top layer, to minimize diffusion of CO_2 through the silicone oil into the bottom layer. The amount of CO_2 which diffused through the silicone layer was less than 5% of the measured CO_2 pool over the entire range of CO_2 concentrations. As soon as possible after centrifugation, the tips of the plastic tubes were frozen in liquid N_2 and the bottom alkaline layer was removed by cutting and placed in a vial containing 430 μl of 0.1 N NaOH.

Measurement of (CO_2 + HCO_3^-). After thawing, the pellet was resuspended in the liquid and two 170-μl samples were taken. One sample was placed in an equal volume of 0.1 N NaOH and the other, in an equal volume of 0.5 N HCl. The acidified sample was allowed to sit at room temperature for 30 min, with frequent flushing with CO_2. Radioactivity was determined in each sample by liquid scintillation counting. The total CO_2 pool (CO_2 + HCO_3^-) was estimated from the difference between the alkaline- and acid-stable counts. That the pool sizes measured in both cell types represent only acid-unstable inorganic carbon was checked by: (*a*) collection of $^{14}CO_2$ released from the acidified samples on filter papers soaked with 0.1 N KOH (the counts collected were within 10% of the values estimated by the difference technique); and (*b*) attempts to reduce the unstable counts by stabilization of keto acids as the hydrazones. This was done by addition of 1% (w/v) hydrazine HCl to all solutions. The estimated pool sizes were not altered by this procedure.

Measurement of Photosynthesis. Fixation of CO_2 was determined from the acid-stable counts measured during the same experiments in which the CO_2 pool was estimated.

Measurement of Cell Volume. The intracellular water space and the free water taken down with the cells, through the silicone oil, was estimated using 5 μM [^{14}C]sorbitol and [^3H_2O]. Separate incubations with each were done for 20 s, by which time equilibrium had occurred (as determined from the time course). The ^{14}C and ^{3}H in the pellet were determined by liquid scintillation counting. The intracellular space (sorbitol-impermeable) was estimated as the total water volume minus the sorbitol-permeable space volume. Three replicates of each incubation were made to determine the average volumes.

Measurement of Internal pH. The intracellular pH was measured by the distribution of [^{14}C]DMO (8). Incubation with DMO in the light was found to reach equilibrium distribution between inside and outside by 45 s, after which time total ^{14}C spun down with the cells remained constant for several min. Thus, pH was estimated from incubations ranging from 45 to 90 s. The internal pH was calculated from the known external pH, external (DMO), and the measured internal (DMO). Four replicates were made for each determination. For estimation of internal pH after the cells had been treated with the inhibitors methyl viologen, DCMU, SF6847, and ethoxzolamide, the addition of DMO and inhibitor occurred at approximately the same time.

RESULTS

Time Course of [CO_2 + HCO_3^-] Accumulation and Photosynthesis. The changes with time in internal [CO_2 + HCO_3^-] (acid-labile ^{14}C) and total photosynthesis (acid-stable ^{14}C) after addition of $NaH^{14}CO_3$ to both low and high CO_2-grown cells of *Chlamydomonas* are presented in Figure 1. Experiments were performed at the indicated external inorganic carbon concentration, and cell density, so that the external [CO_2 + HCO_3^-] did not decrease more than 10% during the longest incubation. At the external and internal pH values encountered in these experiments, the acid-labile pool should be composed of CO_2 and HCO_3^- species. Thus, the total calculated pool size is referred to as [CO_3 + HCO_3^-]. In this and the following experiments, the inorganic carbon concentration and photosynthesis were calculated on the basis of internal water volume or SIS.

In low CO_2 cells, the internal [CO_2 + HCO_3^-] rapidly reached

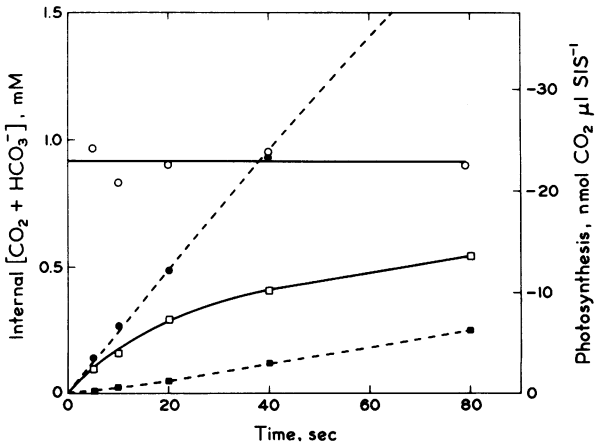

FIG. 1. The change of internal [CO_2 + HCO_3^-] (——) and total photosynthesis (– – –), with time of incubation, for both high (□) and low (○) CO_2-grown cells. Incubations were in 25 mM Hepes (pH 7.15, 25 C) and 100 μM $NaH^{14}CO_3$. Three replicates were run for each time point. The sample of high CO_2 cells for each replicate represented 0.91 μl SIS and 2.00 μl total water space. For the low CO_2 cells, there was 0.25 μl SIS and 0.75 μl total water space. Internal pH values were estimated as described in Figure 4, except that 100 μM $NaHCO_3$ was included. The values obtained were pH 7.10 (high CO_2) and pH 7.05 (low CO_2). Photosynthesis is expressed on a SIS basis.

an equilibrium level which remained essentially unchanged for the entire time course. Such a rapid equilibrium situation is consistent with a fast interconversion of CO_2 and HCO_3^-, catalyzed by carbonic anhydrase. Photosynthesis showed no lag in the time course of CO_2 fixation. The rate over the first min of 35.1 nmol μl^{-1} SIS min^{-1} corresponds to 334 μmol mg^{-1} Chl h^{-1} (from Chl determinations on cells used in Fig. 1). This rate, at 100 μM [CO_2 + HCO_3^-] should be approaching a CO_2-saturated value (Fig. 2) and is comparable to the rates of O_2 evolution obtained in long-term steady-state experiments (Fig. 6). These observations indicate that the time required to reach steady-state was short in relation to the shortest time intervals measured in this and the following experiments. Thus, it is assumed that the measurements reported here using low CO_2-grown cells are representative of steady-state photosynthetic performance.

For high CO_2 cells, on the other hand, there is a gradual increase in the internal [CO_2 + HCO_3^-] with time and this appears to be correlated with an increasing rate of photosynthesis. This slow increase in pool size suggests that there is not rapid equilibrium between CO_2 and HCO_3^- within these cells. This is in contrast to the situation for low CO_2-grown cells. This difference may be the result of the comparatively low level of carbonic anhydrase in high CO_2-cells (6, 7, 17). It is also apparent from the time course that the initial rate of inorganic carbon accumulation in the high CO_2-grown cells is much slower than for the low CO_2-grown cells.

Response of Internal [CO_2 + HCO_3^-] to External [CO_2 + HCO_3^-]. From the experiments in Figure 1, it is possible to use the estimated internal pH, calculated using DMO, to determine the internal [CO_2 + HCO_3^-] expected, assuming the cell membrane is permeable to CO_2 and impermeable to HCO_3^-, and that rapid equilibrium occurs between CO_2 and HCO_3^-. The pH calculated by this DMO technique represents an average cell pH, as the cytoplasm will be a different pH to that of the chloroplast. However, the distribution of DMO should respond passively, according to the pH gradients, in the same manner as inorganic carbon species (23). Thus the pH estimated from DMO distribution will be valid in calculating the passive distribution expected for CO_2 and HCO_3^-.

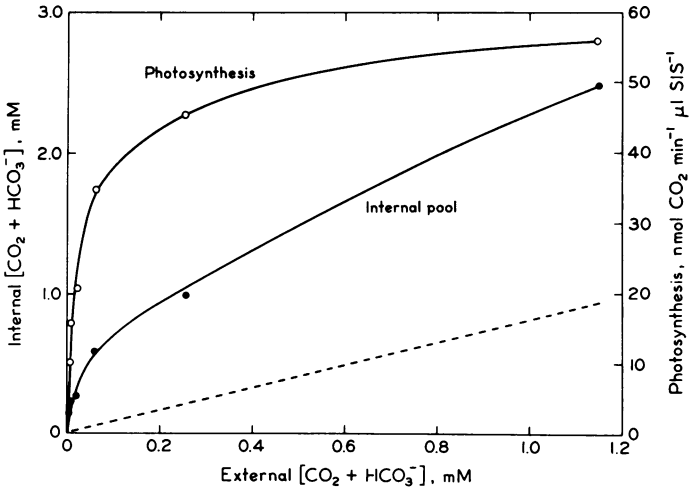

FIG. 2. The response of internal [CO_2 + HCO_3^-] and rate of photosynthesis to external [CO_2 + HCO_3^-] in low CO_2-grown *C. reinhardtii*. Cells were incubated in 25 mM Hepes (pH 7.15, 25 C). The internal pool and photosynthesis were estimated from three 10-s incubations at each external concentration. Each replicate represented 0.41 μl SIS, 1.05 μl total water space, 2.68 μg Chl, and 2.67 × 10^6 cells. The internal pH (7.08) was estimated as described in Figure 4. - - -, internal [CO_2 + HCO_3^-] which would be expected assuming passive distribution of inorganic carbon species according to the estimated internal pH. Photosynthesis is expressed on a SIS basis.

The internal pH values in both low and high CO_2-grown cells, in the experiment shown in Figure 1, vary only slightly from the external medium; thus, it can be roughly calculated that, at passive equilibrium, the internal [CO_2 + HCO_3^-] should equal the external [CO_2 + HCO_3^-]. This is not true for either low or high CO_2 cells. In low CO_2-grown cells, there is approximately at 10-fold higher internal [CO_2 + HCO_3^-] throughout the time course, whereas in high CO_2 cells, this is approximately 1-fold at 5 s to 5-fold at 70 s. Thus, it appears that both cell types are capable of concentrating inorganic carbon in excess of that possible by the measured pH gradient.

To investigate the relationship between external and internal [CO_2 + HCO_3^-], it is essential that an equilibrium relationship between internal and external pool sizes be reached. Furthermore, to make valid comparisons between the measured pool level and the expected passive distribution, it is necessary that CO_2 and HCO_3^- be in rapid equilibrium within the cell. These criteria would appear to be satisfied only in low CO_2-grown cells. Therefore, the response of internal [CO_2 + HCO_3^-] to external [CO_2 + HCO_3^-] was studied only in these cells, and the results are presented in Figure 2.

If the accumulation of CO_2 and HCO_3^-, within the cell, followed a passive distribution in response to a constant pH gradient, then a linear relationship between internal [CO_2 + HCO_3^-] and external [CO_2 + HCO_3^-] would be expected. This passive relationship, calculated for the measured internal pH, is presented in Figure 2. The measured response in low CO_2 cells is distinctly nonlinear and more closely approximates a hyperbola over the tested external concentration range. Such a response could be in part due to a changing internal pH with increasing external [CO_2 + HCO_3^-]. Measurements of internal pH indicate, however, that the pH may increase marginally as external inorganic carbon is increased from 0 to 1 mM (data not shown). This change is in the opposite direction to that necessary to explain the observed accumulation response. Figure 2 also shows that low CO_2-grown cells are capable of concentrating inorganic carbon with respect to the expected passive distribution. As CO_2 and HCO_3^- appear to be in rapid equilibrium within these cells, inorganic carbon accumulation will result directly in an elevation of internal CO_2 above that existing externally. It seems that a CO_2-concentrating mechanism exists in low CO_2-grown cells. The photosynthetic CO_2 response, also shown in Figure 2, is almost saturated by 250 M external [CO_2 + HCO_3^-]. Thus, physiologically the accumulation response is most important in increasing the rate of photosynthetic CO_2 fixation when external [CO_2 + HCO_3^-] is below this concentration.

Energy Dependence of Inorganic Carbon Accumulation. Since CO_2 is accumulated against an apparent concentration gradient (Fig. 2), the mechanism which drives this must be an active process requiring energy. Hence, it would be expected that the concentrating capacity of the system could be reduced by certain energy inhibitors. Thus, the effects of methyl viologen, DCMU, FCCP, and SF6847 on the internal inorganic carbon pool were examined.

Methyl viologen should inhibit NADP reduction within the chloroplast without decreasing the ATP pool within the chloroplast. Thus, it should decrease the carboxylation rate without altering the potential ATP energy supply for the concentrating mechanism (if transport is not directly linked to NADPH). DCMU will inhibit NADP reduction, photosynthetic electron transport, and ATP synthesis within the chloroplast. Hence, availability of photosynthetic energy in all forms will be reduced. FCCP and SF6847 are uncouplers which act by dissipating the proton gradient across membranes (21). These inhibitors should act to stop ATP synthesis in both the chloroplast and mitochondrion.

The effects of these inhibitors were tested on time courses of internal [CO_2 + HCO_3^-] and photosynthesis, in both low and high CO_2-grown cells and the results are shown in Figures 3 and 4, respectively. In low CO_2-grown cells, the effect of each inhibitor

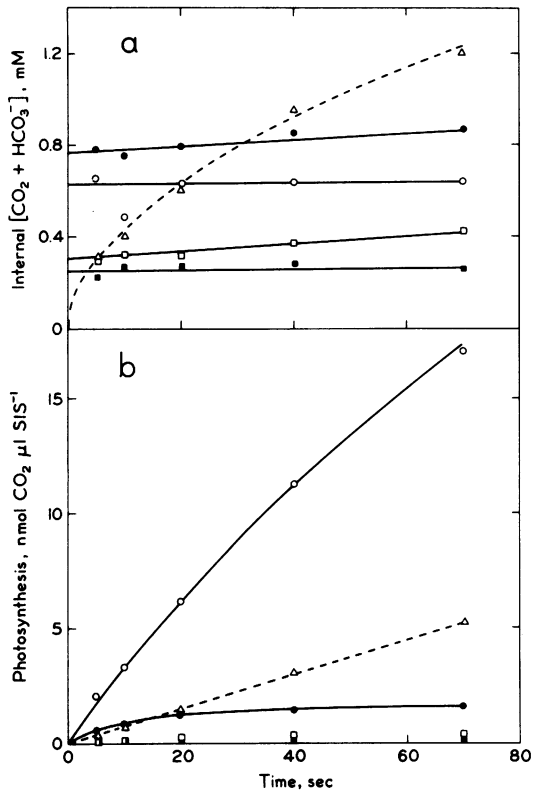

FIG. 3. a: The change of internal $[CO_2 + HCO_3^-]$ with time of incubation in low-CO_2-grown *Chlamydomonas*. Incubations were in 25 mM Hepes (pH 7.15, 25 C) and 60 µM $NaH^{14}CO_3$. The pool sizes at each time point were estimated from three replicates. Time courses are shown for cells treated as follows: (○—○), control; (●—●), 5 mM methyl viologen; (□—□), 10 µM (■—■), 30 µM (△- - -△), 12 µM ethoxyzolamide. Addition of the inhibitors occurred when the cells were transfered from the O_2-electrode chamber into the centrifuge tubes. Cells were incubated in the light with each inhibitor 15 s prior to the addition of $NaH^{14}CO_3$ (at time zero). Preincubation time with each inhibitor was minimized so that unlabeled CO_2 from respiration could not accumulate. In this experiment, the sample of cells for each replicate represented 0.45 µl SIS, 1.23 µl total water space, 3.16 µg Chl, and approximately 3.2×10^6 cells. Internal pH values were estimated by incubation in the light with DMO, 60 µM $NaHCO_3$ and the appropriate treatment for 1 min. Estimated pH values were: control, 7.25; methyl viologen, 7.09; FCCP, 7.03; DCMU, 7.09; ethoxyzolamide, 7.26. b: Time course of acid-stable ^{14}C accumulation (photosynthesis) for the corresponding treatments described in a. This is expressed on a SIS basis.

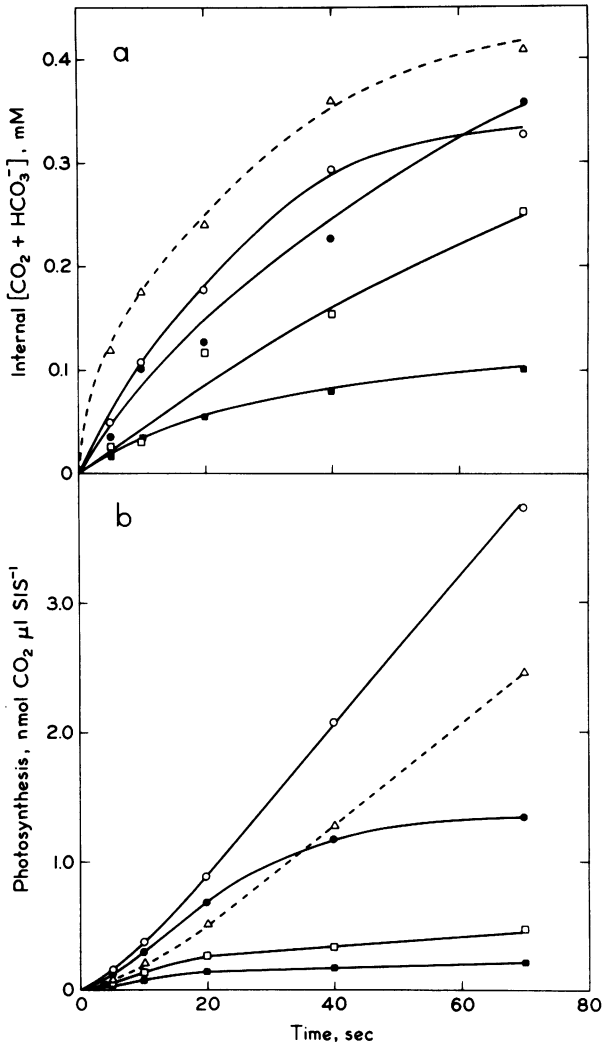

FIG. 4. a: The change of internal $[CO_2 + HCO_3^-]$ with incubation time in high-CO_2-grown *Chlamydomonas*. Incubation conditions and treatments were as described in Fig. 4. (○—○), control; (●—●), 5 mM methyl viologen; (□—□), 10 µM DCMU; FCCP; (■—■), 30 µM (△- - -△), 12 µM ethoxyzolamide. The sample of cells for each replicate represented 1.29 µl SIS, 2.19 µl total water space, 2.52 µg Chl, and approximately 53.5 $\times 10^6$ cells. Internal pH values were estimated by incubation in the light with DMO, 60 µM $NaHCO_3$, and the appropriate treatment for 1 min. Estimated pH values were: control, 7.22; methyl viologen, 7.16; FCCP, 7.00; DCMU, 7.01; ethoxyzolamide, 7.16. b: The course of acid-stable ^{14}C accumulation (photosynthesis) for the corresponding treatments described in a. This is expressed on a SIS basis.

on internal $[CO_2 + HCO_3^-]$ is almost maximal at the shortest measurable time points (Fig. 3a) and remains essentially unchanged throughout the time course. This is correlated with a rapid inhibition of photosynthesis (Fig. 3b). In high CO_2-grown cells, the time courses with the inhibitors show the gradual increase in internal $[CO_2 + HCO_3^-]$ with time seen in Figure 1. The inhibitors appear to affect both the size of the internal pool and the initial rate of entry of inorganic carbon into the cells.

The effect of the above inhibitors on the response of internal $[CO_2 + HCO_3^-]$ to external $[CO_2 + HCO_3^-]$ was investigated in low CO_2-grown cells (Fig. 5). The effect of the inhibitors are maximal by the 10-s incubation times used in this experiment (Fig. 3); thus, for all treatments it may be assumed that steady-state equilibrium levels of internal $[CO_2 + HCO_3^-]$ are measured. In Figure 5a, it is apparent that methyl viologen increases the internal $[CO_2 + HCO_3^-]$ relative to the untreated cells. This can be explained as being due to decreased rate of carboxylation leading to a higher steady-state level of $[CO_2 + HCO_3^-]$ inside the cell. DCMU, FCCP, and SF6847 markedly decrease the ability of the cells to accumulate $[CO_2 + HCO_3^-]$. This can be more clearly seen in Figure 5b. Here the internal pH with each inhibitor has been calculated and used to estimate internal uncharged CO_2 concentration, assuming rapid equilibrium between HCO_3^- and CO_2. The ratio of internal $[CO_2]$ to external $[CO_2]$ then is plotted as a function of external $[CO_2 + HCO_3^-]$, for each treatment.

It is apparent that low CO_2-grown cells are capable of concentrating uncharged CO_2 up to 40-fold with respect to the external medium. This concentration gradient is reduced by FCCP, SF6847, and DMCU so that it more closely approaches a passive distribution. However, although these inhibitors were capable of completely abolishing CO_2 fixation, they did not eliminate the entire concentration gradient, particularly at the lowest external $[CO_2 + HCO_3^-]$ levels. The inhibition patterns in Figure 5 are

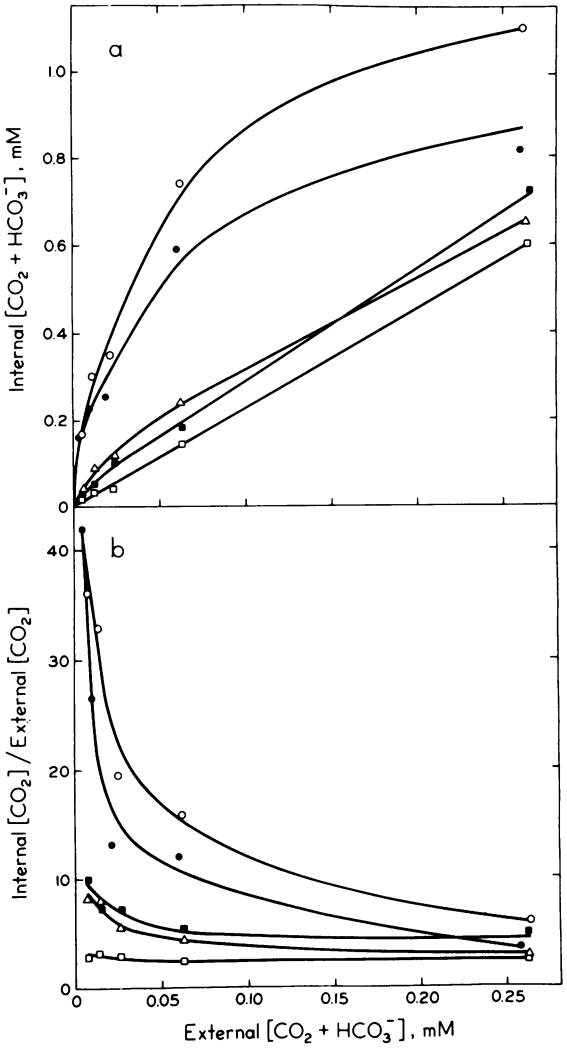

FIG. 5. a: The response of internal [CO_2 + HCO_3^-] to external [CO_2 + HCO_3^-] of low CO_2-grown *Chlamydomonas*, with the following treatments: (○——○), control; (●——●), 5 mM methyl viologen; (■——■), 10 μM DCMU; (△——△), 30 μM FCCP; (□——□), 0.1 μM SF6847. Internal pools were estimated from three 10-s incubations at each external concentration. Treatments with each inhibitor, prior to addition of $NaH^{14}CO_3$, was as described in Figure 3. Each replicate's volume and Chl parameters were as described in Fig. 3. The estimated internal pH values were: control, 7.08; methyl viologen, 7.02; DCMU, 7.07; FCCP, 7.20; SF6847, 6.92. b: The data in a replotted as internal [CO_2]/external [CO_2] against external [CO_2 + HCO_3^-]. Internal [CO_2] was calculated from the internal pH and internal [CO_2 + HCO_3^-]. External [CO_2] was calculated from external pH and [CO_2 + HCO_3^-]. A pK′ of 6.37 (25 C) was assumed.

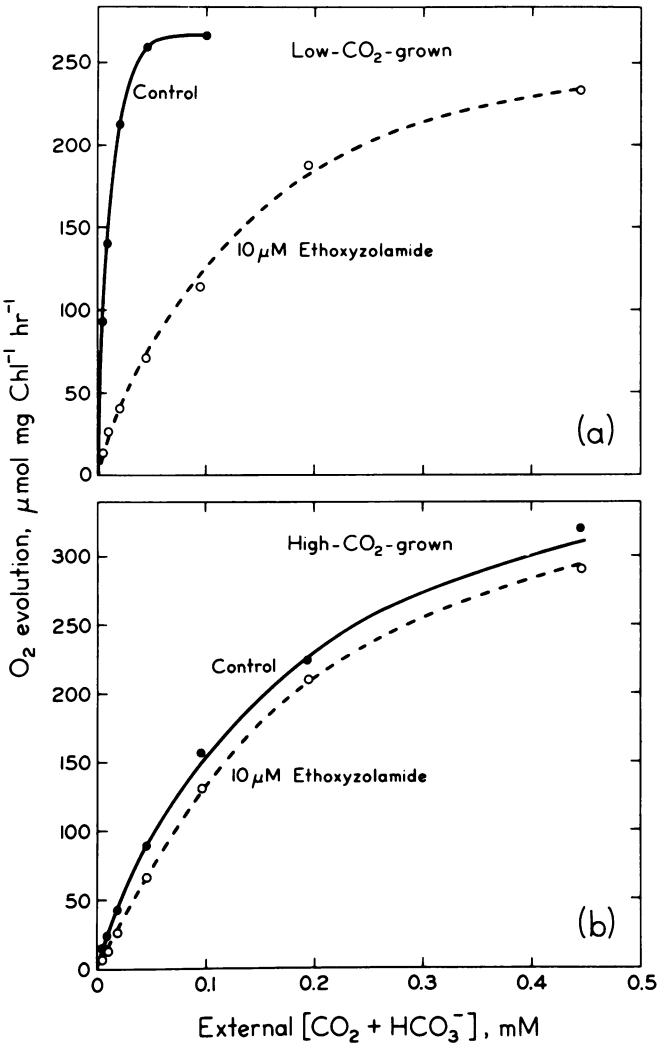

FIG. 6. The effect of ethoxyzolamide (10 μM) on the response of O_2 evolution to external [CO_2 + HCO_3^-], in low (a) and high (b) CO_2-grown cells of *Chlamydomonas*. Measurements were made in an O_2 electrode, 25 C, 50 mM Hepes, (pH 7.15), as described by Berry *et al.* (2).

consistent with those in Figures 3 and 4. The inhibition of the internal pool in high CO_2 cells by FCCP and DCMU is further supportive of the concept that these cells still retain some concentrating capacity.

Effect of Ethoxyzolamide on Internal [CO_2 + HCO_3^-] and Photosynthesis. Increased carbonic anhydrase activity has been shown to be associated with the increased CO_2-assimilation efficiency of low CO_2-grown cells. Ethoxyzolamide is a potent inhibitor of carbonic anhydrase and, when used at concentrations less than 100 μM, has little effect on photosynthetic electron transport (12). The effect of ethoxyzolamide on the response of photosynthetic O_2 evolution to external inorganic carbon is shown in Figure 6. It is apparent that, for low CO_2-grown cells, the inhibitor dramatically decreases the photosynthetic affinity for inorganic carbon, although it does not affect the maximum rate significantly. With high CO_2-grown cells, there appears to be only a slight effect on the overall affinity of photosynthesis, again without an effect on maximum rate. Following this observation, the effect of ethoxyzolamide on the internal [CO_2 + HCO_3^-] of high and low CO_2-grown cells was examined.

The effect of ethoxyzolamide on the time course of internal [CO_2 + HCO_3^-] and photosynthesis is shown in Figures 3 and 4. In low CO_2 cells (Fig. 3), ethoxyzolamide significantly changes the response of pool size with time. A pattern of increasing internal [CO_2 + HCO_3^-] is seen with the final level being 2-fold higher than in untreated cells. This pattern is closely correlated with an increasing rate of photosynthesis, although the absolute rate is very much decreased. In high CO_2 cells (Fig. 4), ethoxyzolamide does not change the pattern of the time course, but it does lead to an increase in the total pool size. The increases in the final pool sizes in both low- and high-CO_2-grown cells cannot be attributed to changes in internal pH as this appears to be similar in treated and untreated cells.

DISCUSSION

The results presented here show that low CO_2-grown cells of *C. reinhardtii* are able to concentrate [CO_2 + HCO_3^-] above the

passive accumulation that would be expected according to the pH gradient. This concentrating ability leads to a nonlinear, hyperbolic response of internal [CO_2 + HCO_3^-] to external [CO_2 + HCO_3^-] up to external concentrations of 1.1 mM (Fig. 2). The time courses in Figures 1 and 3 suggest that within these cells CO_2 and HCO_3^- are in rapid equilibrium. This accumulation of [CO_2 + HCO_3^-] represents a mechanism to concentrate uncharged CO_2 relative to the external medium. Using the measured pH values, it can be seen (Fig. 5) that the concentration of CO_2 inside the cells may be up to 40-fold higher than the external level. In low CO_2-grown cells, photosynthesis increases approximately in parallel with the intracellular CO_2 concentration (Fig. 2). The apparent K_m expressed in terms of external [CO_2 + HCO_3^-] concentration was 35 to 40 μM (or 3.5 to 4.0 μM expressed on the basis of CO_2 concentration). At this external [CO_2 + HCO_3^-] concentration, the internal CO_2 concentration was 30 to 40 μM. This value is approximately 10-fold higher than that of the medium, and similar to the $K_m(CO_2)$ of Ru-P_2 carboxylase from *C. reinhardtii* (2). These results, therefore, reconcile the large difference in apparent $K_m(CO_2)$ of the cells and the isolated enzyme. By concentrating CO_2 (the inorganic carbon substrate for RuP_2 carboxylase [3]), low CO_2-grown cells may increase their apparent photosynthetic affinity for external CO_2.

The properties of low and high CO_2-grown cells, which lead to the differences in their photosynthetic affinities for CO_2, are not as obvious as comparative measurements of internal [CO_2 + HCO_3^-] may suggest. It is apparent from both Figures 1 and 4 that high CO_2-grown cells do possess an ability to concentrate [CO_2 + HCO_3^-]. However, these time courses indicate that there may be slow interconversion of CO_2 and HCO_3^- within the cells. It is impossible to calculate the relative distribution between HCO_3^- and CO_2 and to compare the relative abilities of low and high CO_2-grown cells to concentrate CO_2. It is apparent, however, from the time courses shown in Figures 1 and 4, that the initial rate of accumulation of inorganic carbon is much slower in the high CO_2-grown cells. If the CO_2-concentrating system is based on an inorganic carbon transport system, then this initial accumulation rate may be a measure of the relative operation of the "CO_2-concentrating" system in the two cell types. The observation that the inhibitors DCMU and FCCP reduce both the initial accumulation rate and the final pool size (Fig. 4) in high CO_2-grown cells supports this interpretation. Thus, the lower photosynthetic affinity of high CO_2-grown cells may be most readily explained by a reduction in the capacity for transport of inorganic carbon into the cell. It also appears likely that there may not be discrete physiological differences between high and low CO_2-grown cells but that there may be a continuum of possible states, with the capacity for inorganic carbon transport increasing as supply decreases. This would lead to an increase in the affinity for CO_2 as external levels declined.

The hypothesis of Berry *et al.* (2) that the CO_2 concentrating mechanism may be a HCO_3^- influx pump has descended from the long line of evidence that algae are capable of directly using HCO_3^- from the external medium for photosynthesis (18). Previous studies on HCO_3^- usage had not correlated this with studies of the kinetics of CO_2 assimilation. Only with the recent work by Berry *et al.* (2) on *C. reinhardtii* and Findenegg (4) on *Scenedesmus obliquus* has it become apparent that the ability to transport HCO_3^- into the cell is associated with an overall increased affinity for CO_2. It has been suggested (16) that, in *Chlorella vulgaris*, CO_2 was the active species taken up for photosynthesis, regardless of whether the cells were grown at high or low CO_2. It is unlikely that *Scenedesmus* and *Chlamydomonas* utilize bicarbonate for their CO_2 concentrating mechanisms and that *Chlorella* uses CO_2. It is more likely that a common system exists. The final resolution between CO_2 and HCO_3^-, or even perhaps H_2CO_3, as the actively accumulated species remains to be clearly shown.

The concentrating mechanism is dependent upon an energy supply as shown by the accumulation of CO_2 in excess of that expected by passive distribution (Figs. 2 and 5). This energy dependence is further supported by the effects of the inhibitors FCCP, SF6847, and DCMU (Figs. 3–5). The effect of the uncouplers FCCP and SF6847 indicate that energy supply may be directly or indirectly linked to proton motive gradients within the cell (possibly through ATP), whereas DCMU inhibition suggests that there is some direct dependence of the transport system on photosynthetic energy, through photophosphorylation.

The role of carbonic anhydrase in the operation of the concentrating mechanism is uncertain. If the transporter is located on the chloroplast membrane, then its role may be to provide a rapid supply of HCO_3^- externally and to reconvert it to CO_2 once this species is moved into the chloroplast. If transport occurs at the cell membrane, then it would serve as a means of promoting facilitated diffusion of [CO_2 + HCO_3^-] species to the chloroplast. Transport of HCO_3^- is likely to be accompanied by either proton influx or hydroxyl efflux. If this occurs, then an inhibition of carbonic anhydrase would result in alteration in the relationship between proton influx (or hydroxyl efflux) and proton consumption occurring when HCO_3^- is converted to CO_2. This may alter the pH regulation process in the cell and feedback to inhibit HCO_3^- transport. Inhibition of carbonic anhydrase activity by ethoxyzolamide leads to a decrease in the rate of photosynthesis in both low and high CO_2-grown cells. Such inhibition occurs only at subsaturating CO_2 levels where the transport system is needed to increase the [CO_2] at the site of carboxylation. The inhibition is much higher in low CO_2 cells, presumably because of the greater transport capacity of these cells.

The effects of ethoxyzolamide on the internal pool size are not easily interpreted without a clear understanding of how carbonic anhydrase is involved in the concentrating mechanism. The increase in internal [CO_2 + HCO_3^-], caused by this inhibitor in both cell types (Figs. 3 and 4), is not readily explained if CO_2 is the transported species. This would mean that CO_2 would be higher in the treated cells and photosynthesis should not be inhibited. The alternative of HCO_3^- transport provides for the possibility that slow interconversion between HCO_3^- and CO_2 will lead to the situation where HCO_3^- is high, whereas CO_2 is low, and the internal inorganic carbon pool will appear to increase. Inhibition of the initial rate of inorganic carbon accumulation by ethoxyzolamide is evident for low CO_2-grown cells in Figure 3. This may be due to either some direct effect of the inhibitor on the transport system or possibly to the fact that transport occurs at the chloroplast membrane, and thus can be limited by the interconversion of CO_2 to HCO_3^- within the cytoplasm.

A mechanism for concentrating CO_2 represents a means by which the overall rate of photosynthesis can be increased at ambient levels of CO_2. The increase would be a result of both the direct effect of CO_2 on the rate of carboxylation by Ru-P_2 carboxylase, and an indirect effect of the suppression of Ru-P_2 oxygenase by elevated CO_2 concentrations. Thus, this is documentation of another mechanism, in addition to the C_4 pathway, evolved by photosynthetic organisms to increase photosynthetic competence without altering the molecular properties of Ru-P_2 carboxylase.

Glycolate synthesis, its excretion and metabolism, has been studied in several unicellular algae and cyanobacteria (2, 5, 9, 11, 22). The main conclusion which can be drawn from this work is that both low- and high-CO_2-grown algae produce and excrete glycolate. However, the range of external CO_2 concentrations over which glycolate can be shown to be synthesized is where CO_2 is limiting to photosynthesis. This would be expected if Ru-P_2 oxygenase activity is the main source of glycolate and is suppressed as [CO_2] is increased (1, 14). It has also become apparent that many green and blue-green algae lack what is commonly called photorespiration and an O_2 effect on photosynthesis (15, 19). This

would be consistent with a more general capacity of these algae to concentrate CO_2 in the same manner as *C. reinhardtii.* It is reasonable to expect that any photosynthetic algae, bacteria, or cyanobacteria which shows the high- to low-CO_2 induction phenomenon, characterized by increases in carbonic anhydrase and increased capacity to photosynthesize at low CO_2, would possess this mechanism to concentrate CO_2.

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