Active CO₂ Transport by the Green Alga
Chlamydomonas reinhardtii

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ABSTRACT

Mass spectrometric measurements of dissolved free 13CO₂ were used to monitor CO₂ uptake by air grown (low CO₂) cells and protoplasts from the green alga Chlamydomonas reinhardtii. In the presence of 50 micromolar dissolved inorganic carbon and light, protoplasts which had been washed free of external carbonic anhydrase reduced the 13CO₂ concentration in the medium to close to zero. Similar results were obtained with low CO₂ cells treated with 50 micromolar acetazolamide. Addition of carbonic anhydrase to protoplasts after the period of rapid CO₂ uptake revealed that the removal of CO₂ from the medium in the light was due to selective and active CO₂ transport rather than uptake of total dissolved inorganic carbon. In the light, low CO₂ cells and protoplasts incubated with carbonic anhydrase took up CO₂ at an apparently low rate which reflected the uptake of total dissolved inorganic carbon. No net CO₂ uptake occurred in the dark. Measurement of chlorophyll a fluorescence yield with low CO₂ cells and washed protoplasts showed that variable fluorescence was mainly influenced by energy quenching which was reciprocally related to photosynthetic activity with its highest value at the CO₂ compensation point. During the linear uptake of CO₂, low CO₂ cells and protoplasts incubated with carbonic anhydrase showed similar rates of net O₂ evolution (102 and 106 micromoles per milligram of chlorophyll per hour, respectively). The rate of net O₂ evolution (83 micromoles per milligram of chlorophyll per hour) with washed protoplasts was 20 to 30% lower during the period of rapid CO₂ uptake and decreased to a still lower value of 46 micromoles per milligram of chlorophyll per hour when most of the free CO₂ had been removed from the medium. The addition of carbonic anhydrase at this point resulted in more than a doubling of the rate of O₂ evolution. These results show low CO₂ cells of Chlamydomonas are able to transport both CO₂ and HCO₃⁻ but CO₂ is preferentially removed from the medium. The external carbonic anhydrase is important in the supply to the cells of free CO₂ from the dehydrogenation of HCO₃⁻.

Green algae and cyanobacteria possess a high apparent affinity for DIC when grown at low DIC concentrations (low CO₂ cells: 2, 5, 9, 17), and DIC accumulation has been demonstrated for these organisms (2, 4, 9, 15). In the case of cyanobacteria, both HCO₃⁻ and CO₂ are substrates for active transport (2, 3, 6, 7, 14, 15) with CO₂ being selectively and preferentially used by the cells (2, 6, 16). In Chlamydomonas, HCO₃⁻ is actively transported (4, 25, 29), but CO₂ uptake has been considered to be passive (18, 20). Carbon dioxide, however, is taken up from the medium faster than HCO₃⁻ by Chlamydomonas (13, 28, 29) and several authors (13, 29) have considered the possibility of active CO₂ transport.

Studies on the DIC transport mechanism of green algae are complicated by their cellular compartmentation. Recently, it was shown that isolated chloroplasts of low CO₂ Chlamydomonas reinhardtii were able to accumulate DIC (19) and a model was presented where the only active DIC transport mechanism was located on the chloroplast envelope (18, 19). In that model the plasma membrane was suggested to be only a diffusion barrier for CO₂ generated by external carbonic anhydrase. In contrast, by comparison of the apparent affinities for DIC of whole cells and purified chloroplasts, Süttemeyer et al. (26) came to the conclusion that active transport by the chloroplast alone may not be responsible for the photosynthetic characteristics of whole cells.

Another difficulty in examining the DIC species taken up by whole cells is the presence of an external carbonic anhydrase (10) which catalyzes the rapid equilibrium between CO₂ and HCO₃⁻, thus making a direct discrimination between CO₂ and HCO₃⁻ uptake impossible (7, 13). However, using inhibitors for external carbonic anhydrase or the cell-wall less mutant CW-15, some authors came to the conclusion that CO₂ and not HCO₃⁻ (13) or that both CO₂ and HCO₃⁻ (29) were actively transported.

Confusion about which DIC species is actively taken up from the medium may also be caused by methods which only measure total rates of transport rather than transport of CO₂ or HCO₃⁻ individually. Using MS, which measures free dissolved gases in liquid, several authors presented direct evi-
dence consistent with an active CO₂ transport in cyanobacteria (2, 6, 16). We have used mass spectrometry (2, 16) for measuring CO₂ and O₂ exchange in C. reinhardtii. To overcome the problem of external carbonic anhydrase we used protoplasts of low CO₂ C. reinhardtii which were washed free of external carbonic anhydrase activity. The results presented in this paper will show that both CO₂ and HCO₃⁻ are actively taken up from the medium but that CO₂ is preferentially used. Further, we shall present evidence that the function of external carbonic anhydrase is to supply the cells with free CO₂.

MATERIALS AND METHODS

Growth of Algae

Chlamydomonas reinhardtii (Dangeard) strain 11/32b (obtained from the Sammlung für Algenkulturen, Göttingen, FRG) was grown in 50 mL glass tubes at 32°C in a medium described by Kreuzberg et al. (12), except that 50 mM TAPS was added in order to maintain pH 8.0. The cultures were either gassed with air (low CO₂ cells) or with air enriched with 5% CO₂ (high CO₂ cells). During growth, the algae were continuously illuminated by a PFR of 150 μmol photons m⁻² s⁻¹.

Preparation of Cells and Protoplasts

For each measurement, an aliquot of algae containing about 30 μg of total Chl was harvested and washed three times by centrifugation in 1 mL of 25 mM BTP/HCl buffer (pH 8.0). This buffer contained only small amounts (15 μM) of DIC (14).

For preparations of protoplasts, an aliquot of low CO₂ grown cells (total Chl about 30 μg) was harvested, washed in 25 mM Hepes KOH (pH 7.8), and incubated in 5 mL of autolysin which was prepared according to Schlösser et al. (22). At room temperature protoplast formation was completed within 10 min. Isolated protoplasts were washed six times in 1 mL BTP/HCl-buffer (pH 8.0) to ensure complete removal of external carbonic anhydrase activity (Fig. 1).

Fluorometry

Fluorescence measurements were carried out with a pulse modulated Chl fluorometer (24) at the same time as O₂ and ¹³CO₂ exchanges were measured by MS. The pulsed measuring beam (100 kHz) and the actinic light to drive ¹³CO₂ uptake and O₂ evolution were about 1 and 220 μmol photons m⁻² s⁻¹, respectively. The latter was provided by a tungsten-halogen projector lamp. The oxidation state of Q was monitored at 10 s intervals with a high intensity light flash (1600 μmol photons m⁻² s⁻¹) for 1 s. F₂max was obtained by pulsing dark adapted cells with one single high intensity light flash and the quenching coefficients for photochemical (qϕ) and nonphotochemical (qₑ) fluorescence quenching were calculated using the formulas of Schreiber et al. (24).

Mass Spectrometry

The uptake of ¹³CO₂ (m/e = 45) and the release of O₂ (m/e = 32) by cells and protoplasts were continuously recorded using the closed system described by Miller et al. (16). It consists of a water-jacketed cuvette which is coupled to a magnetic sector MS (VG Gas Analysis, model MM 14-80 SC; Middlewich, England) via an aqueous membrane inlet system.

Washed cells and protoplasts were resuspended in 6 mL BTP/HCl-Buffer (pH 8.0; final Chl content 4–6 μg mL⁻¹) and introduced into the cuvette thermostated at 30°C. The chamber was closed with a plexiglas stopper which contained a small capillary and was darkened for temperature equilibration. The suspension was continuously stirred during the measurements.

Measurements of Carbonic Anhydrase

The activity of external carbonic anhydrase was monitored by MS by following the time course of ¹³CO₂ formation after addition of K₄[¹³CO₃]. The chamber was filled in the dark at 30°C with 6 mL of BTP/HCl buffer (pH 8.0) containing the cell material (final Chl concentration = 4–6 μg mL⁻¹) and closed. Subsequently, 3 μL of K₂[¹³CO₃] stock solution (100 mM) were added to yield an initial concentration of 50 μM and the increase in the signal at mass 45 (¹³CO₂) was followed. For a further comparison, buffer without cells was measured either in the absence or presence of bovine carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹).

Chemicals

Bovine carbonic anhydrase and BTP were obtained from Sigma Chemical Co. K₂[¹³CO₃] (99 atom % ¹³C) was purchased from MSD Isotopes, Montreal, Canada. Other chemicals were of reagent grade.

RESULTS

The possibility of measuring selective CO₂ uptake by mass spectrometry (2, 16) in low CO₂ grown C. reinhardtii is complicated by the presence of an external carbonic anhydrase (trace b, Fig. 1). This activity could be abolished by addition of 50 μM AZA, a potent inhibitor of carbonic anhydrase (trace e, Fig. 1) where the rate of CO₂ formation was similar to that in BTP buffer without cells (trace c, Fig. 1). Similar to AZA treated low CO₂ cells, high CO₂ grown algae and washed protoplasts from low CO₂ grown C. reinhardtii did not show any measurable carbonic anhydrase activity (traces d and f, Fig. 1). The initial rates of CO₂ appearance in traces c to f (Fig. 1) showed a rate constant K₀ of 0.88 × 10⁻³ s⁻¹, which is in close agreement to values reported previously (6, 16) for the uncatalyzed dehydration of HCO₃⁻.

To examine the ability for CO₂ uptake, cells and protoplasts were illuminated after being incubated in the dark for several minutes with 50 μM K₂[¹³CO₃] (0.8 μM ¹³CO₂ at pH 8.0). High CO₂ cells could use the inorganic carbon only to a small degree (trace d, Fig. 2), whereas low CO₂ algae were more effective (trace b, Fig. 2) indicating a higher affinity for DIC. This is supported by the Kₘ (DIC) values which were 12 μM DIC (0.19 μM CO₂ at pH 8.0) and 332 μM DIC (5.2 μM CO₂ at pH 8.0) for low and high CO₂ cells, respectively (not shown). When 50 μM AZA was added to low CO₂ cells to inhibit the
low CO₂ cells by the addition of bovine carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹; trace g, Fig. 2).

In order to investigate the nature of the rapid ¹³CO₂ disappearance from the external medium, further experiments were conducted with washed protoplasts from low CO₂ cells. Complications due to AZA, such as possible inhibition of intracellular carbonic anhydrase (29) could thus be avoided.

After a period of ¹³CO₂ uptake in the light, darkening of the protoplasts resulted in a reappearance of ¹³CO₂ in the medium (Fig. 3A). Estimation of the velocity by which ¹³CO₂ was formed after darkening (Fig. 3A) led to a k₀ value similar to that for spontaneous dehydration of HCO₃⁻ to CO₂ (Fig. 1). Thus, the reappearance of ¹³CO₂ in the medium upon darkening was due mainly to reequilibration of the HCO₃⁻/CO₂ system. Addition of carbonic anhydrase in the light caused a rapid increase in the ¹³CO₂ concentration in the external medium (Fig. 3B) indicating a disequilibrium of the HCO₃⁻/CO₂ system prior to carbonic anhydrase addition similar to that observed in cyanobacteria (6, 16). Because the fixation period (1.5 min) was short, dark respiration was not likely to contribute to the reappearance of ¹³CO₂ and because of the kinetics in the absence and presence of carbonic anhydrase, leakage of ¹³CO₂ from the internal pool could at most make a minor contribution to the reappearance of ¹³CO₂.

Since carbonic anhydrase catalyzes a fast equilibration between HCO₃⁻ and CO₂, it appears from these results that at least one function of carbonic anhydrase is to ensure the availability of CO₂. In order to check this hypothesis, we compared the O₂ evolution, CO₂ uptake, and fluorescence quenching patterns of whole cells and protoplasts (Figs. 4–6).

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**Figure 1.** Time course of ¹³CO₂ formation in the dark after injection of 50 μM K₂¹³CO₃ (DIC) as measured by mass spectrometry at 30°C and pH 8.0. a, BTP-buffer + carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹); b, low CO₂ grown cells; c, 25 mM BTP/HCl buffer; d, high CO₂ grown cells; e, low CO₂ grown cells treated with 50 μM AZA; f, washed protoplasts from low CO₂ grown cells.

**Figure 2.** Measurement of ¹³CO₂ uptake by mass spectrometry during illumination at pH 8.0 in the presence of 50 μM K₂¹³CO₃ (0.8 μM ¹³CO₂). b, Low CO₂ grown cells; d, high CO₂ grown cells; e, AZA (50 μM) treated low CO₂ grown cells; f, washed protoplasts from low CO₂ grown cells; g, washed protoplasts from low CO₂ grown cells incubated with carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹).

**Figure 3.** Time courses of ¹³CO₂ uptake by washed protoplasts of low CO₂ cells of *C. reinhardtii*. Protoplasts were incubated with 50 μM K₂¹³CO₃ (0.8 μM ¹³CO₂) for several minutes in the dark. The transport of ¹³CO₂ was induced by switching the light on as indicated (L). Reequilibration was achieved by: A, dark; B, addition of carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹). Each horizontal and vertical bar represents 10 s and 0.16 μM ¹³CO₂, respectively.
In the presence of 50 μM DIC, low CO₂ cells reached CO₂ compensation (about 50–60 nM CO₂) within 8 min following illumination (Fig. 4). This was indicated by the cessation of O₂ evolution and CO₂ uptake. Simultaneously, the nonphotochemical quenching (qₑ) drastically increased from 0.2 (at maximal photosynthesis) to 0.76 (at CO₂ compensation) which is indicative of an enhanced thylakoid membrane energization at CO₂ limitation (24). The photochemical quenching (qₒ) was not affected over the same time period.

In the presence of carbonic anhydrase, rapid equilibration between HCO₃⁻ and CO₂ occurs, and the [CO₂] measured by MS then represents the uptake of total DIC. At a known pH (pH = 8.0), it is possible to calculate rates of DIC uptake from the decline of the CO₂ trace. DIC uptake was 93 μmol mg⁻¹ Chl h⁻¹ and O₂ evolution was 108 μmol mh⁻¹ Chl h⁻¹ in low CO₂ cells (Fig. 4). When protoplasts were incubated with carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹), they exhibited similar rates (93 and 102 μmol mg⁻¹ Chl h⁻¹) of DIC uptake and O₂ evolution (Fig. 5) as low CO₂ cells (Fig. 4). The fluorescence yield pattern in protoplasts with carbonic anhydrase (Fig. 5) was essentially the same as that in whole cells (Fig. 4) showing an increase in qₑ when the CO₂ compensation point was approached. Again, qₒ was little changed.

Figure 6 shows the changes of fluorescence yield and ¹³CO₂ and O₂ concentrations during photosynthesis in the absence and presence of carbonic anhydrase by washed protoplasts. Without carbonic anhydrase, ¹³CO₂ was initially taken up at a rate of about 16 μmol mg⁻¹ Chl h⁻¹ (since CO₂ and HCO₃⁻ were not in equilibrium this rate cannot be expressed in terms of DIC). Simultaneously, O₂ was evolved but at a lower rate (83 μmol mg⁻¹ Chl h⁻¹) than with whole cells and carbonic anhydrase-treated protoplasts (Figs. 4 and 5). Before addition of carbonic anhydrase, the rate of O₂ production had declined to 46 μmol mg⁻¹ Chl h⁻¹. Oxygen evolution increased about 2.5-fold after addition of carbonic anhydrase (Fig. 6). Carbonic anhydrase reequilibrated the CO₂/HCO₃⁻ system and this reequilibration indicated that about one-half of the initial [DIC] (50 μM) had been taken up and fixed by the cells.

As was observed for the CO₂ and O₂ gas exchanges, the fluorescence yield patterns also responded to the addition of carbonic anhydrase (Fig. 6). Without carbonic anhydrase, qₑ was relatively high (qₑ = 0.45) 2 min after onset of illumination (Fig. 6) compared to whole cells and carbonic anhydrase treated protoplasts (Figs. 4 and 5). It slowly increased during CO₂ depletion and cessation of O₂ evolution and reached 0.58

Figure 4. Changes in the fluorescence, ¹³CO₂ concentration and total O₂ content during illumination in low CO₂ cells of C. reinhardtii in the presence of 50 μM DIC. The coefficients qₒ and qₑ are indicated when maximal photosynthesis, declining photosynthesis and CO₂ compensation occurred. The fluorescence yield of dark-adapted cells after the first light flash was taken as Fₚ₉₅₀ according to Schreiber et al. (24). Numbers in parentheses indicate the rates of total DIC uptake and O₂ evolution expressed as μmol mg⁻¹ Chl h⁻¹. Horizontal bars represent 1 min and the vertical bar indicates 0.16 μM CO₂ and 8 μM O₂, respectively.

Figure 5. Changes in fluorescence, ¹³CO₂ concentration and total O₂ content during illumination by washed protoplasts of C. reinhardtii in the presence of 50 μM DIC and carbonic anhydrase (50 Wilbur-Anderson units mL⁻¹). Horizontal bars represent 1 min and the vertical bar indicates 0.16 μM CO₂ and 8 μM O₂, respectively. For further details see Figure 4.
The adaptation of *C. reinhardtii* to low DIC concentrations is accompanied by an increased affinity for DIC (5), which is correlated with increased levels of carbonic anhydrase (10) and an increased ability to actively accumulate DIC (4, 18, 20, 25). Most of the carbonic anhydrase activity that appears is due to increased amounts of extracellular carbonic anhydrase (10). It has been suggested that the function of the external carbonic anhydrase is to provide the cells with CO₂ (8, 18, 20, 28) but photosynthesis could be sustained when the external carbonic anhydrase was inhibited (13, 29). The question of the role of the external carbonic anhydrase is strongly associated with the question of which DIC species is taken up by the cells. Some authors (18–20, 28) propose that both HCO₃⁻ and CO₂ may be actively accumulated (13, 25, 29).

Removal of the extracellular carbonic anhydrase activity is necessary in order to investigate which forms of DIC are transported into the cells (6, 7, 16). This was accomplished by two methods. In the first method the cells were treated with AZA, a potent inhibitor of carbonic anhydrase (Fig. 1, trace e). In the second method protoplasts were made from low CO₂ cells and washed extensively until no carbonic anhydrase activity could be detected (Fig. 1, trace f).

In the absence of external carbonic anhydrase, cells and protoplasts rapidly removed CO₂ from the medium with an initial rate of about 16 μmol CO₂ mg⁻¹ Chl h⁻¹ (Figs. 2, 3, 6). CO₂ uptake occurred faster than CO₂ could be supplied to the medium by HCO₃⁻ dehydration and the CO₂ concentration dropped to the level of the CO₂ compensation point (50–60 nm CO₂). That the CO₂/HCO₃⁻ system was removed from equilibrium was shown by the increase in mass 45 (¹³CO₂) upon addition of carbonic anhydrase (Figs. 3B and 6). Maintenance of the CO₂/HCO₃⁻ system out of equilibrium cannot be explained by simple diffusion of CO₂ across the plasma membrane (18–20, 25, 28) but could only occur by the input of energy, presumably to a CO₂ transport system.

The specific and rapid removal of CO₂ by protoplasts cannot be attributed to passive uptake of CO₂ into a more alkaline compartment because the external pH of 8.0 was higher than the intracellular pH values (7.1–7.5) that have been reported (4, 25), and such a mechanism of initial CO₂ removal could not maintain the CO₂/HCO₃⁻ system out of equilibrium for any length of time. It also cannot be attributed to fixation by Rubisco because with a *K₈*(CO₂) of 57 μM (5) the activity of this enzyme would be slight at the CO₂ concentration of 0.8 μM and of even less significance at the CO₂ compensation point of 50 to 60 nm CO₂. Moreover, high CO₂ cells without carbonic anhydrase activity (Fig. 1) could not remove CO₂ from the medium (Fig. 2) even though the *K₈*(CO₂) of Rubisco in these cells is similar to that in low CO₂ cells and the total activity is higher (5).

The location of active CO₂ transport is not known, and results such as those presented would be obtained if a CO₂ transporter(s) was located either on the plasma membrane or the chloroplast envelope or both. The results could also be obtained through the diffusion of CO₂ to the cytoplasm, rapid conversion to HCO₃⁻, and active transport of HCO₃⁻ at the chloroplast envelope if the rate of transport of HCO₃⁻ at the chloroplast envelope was sufficient to maintain the HCO₃⁻ concentration in the cytoplasm at a level less than 1 μM. Such a low concentration would be required to obtain a cytoplasmic CO₂ concentration that would maintain the diffusion gradient for CO₂ from the external medium where the CO₂ concentration is about 50 nm. Such a mechanism would require an extremely efficient HCO₃⁻ transporter in the chloroplast envelope. While we think this mechanism is unlikely, until the capability of the chloroplast for inorganic carbon transport is known, the possibility cannot be ruled out.

The possibility of active CO₂ transport by *Chlamydomonas* was proposed by Marcus et al. (13) and Williams and Turpin (29). The latter authors demonstrated that low CO₂ cells treated with AZA accumulated DIC up to 25 times the
external DIC concentration. In terms of CO₂, the accumulation was at least 100-fold the external CO₂ concentration assuming an internal pH of 7.5 (25), internal equilibrium between CO₂ and HCO₃⁻, and an external CO₂ content of 0.8 μM. The internal DIC pool reached a maximum value within 30 s after the onset of illumination (29), which is the same as the time course of the rapid CO₂ uptake by AZA-treated cells and protoplasts (Figs. 2, 3, and 6). Thus, the observed CO₂ transport undoubtedly occurred against a concentration gradient. Since there is no reason to assume that the fast CO₂ disappearance of AZA-treated cells is different from that observed with carbonic anhydrase free protoplasts, we conclude from the present data that CO₂ transport is an active uptake mechanism coupled to metabolic energy. Preliminary studies with inhibitors suggest that it is photosynthetic electron flow rather than mitochondrial respiration which is involved in providing the energy for the CO₂ transport (DF Sülttemeyer, unpublished results).

The measurement of fluorescence showed that inorganic carbon transport and fixation primarily affected nonphotochemical quenching (qₑ) rather than photochemical quenching (qₓ) (Figs. 4, 5, and 6). As expected (11, 24) qₑ was smaller when carbon was available for fixation and increased to a high value (0.75) when the inorganic carbon supply was exhausted (Figs. 4, 5, and 6). In the absence (Fig. 6) of carbonic anhydrase, qₑ was larger (0.45) than in the presence (Fig. 5) of carbonic anhydrase (0.16) about 2 min after the start of the experiment, indicating that the DIC was not as effectively used when carbonic anhydrase was not present. The decrease in qₑ to 0.29 when carbonic anhydrase was added to the protoplasts (Fig. 6) supports this view. When the [CO₂] in the medium was close to zero before the addition of carbonic anhydrase (Fig. 6), qₑ only increased to 0.58 and not to 0.75, the value that was observed when all the inorganic carbon had been used (Figs. 4 and 5). This value (0.58) probably reflects not only the use of CO₂ formed from the dehydra-
tion of HCO₃⁻ but also the direct transport and utilization of the HCO₃⁻ ion. The small changes in photochemical quenching (qₓ) are puzzling. The high values of qₓ (0.54–0.75), even at the CO₂ compensation point, indicate that oxidized Q was available and that there still must exist a substantial flow of electrons from it to an oxidant. In the absence of inorganic carbon, a high rate of O₂ uptake was observed in C. reinhardtii (27) suggesting that, in these circumstances, oxygen may be reduced as has been proposed for higher plants (23).

In the absence of carbonic anhydrase, the rates of CO₂ uptake measured with Chlamydomonas (Fig. 6) were similar to those reported for cyanobacteria under similar conditions (6, 16). Unfortunately, it is not yet possible to prevent the use of HCO₃⁻ in Chlamydomonas, and the rate of O₂ evolution (83 μmol mg⁻¹ Chl h⁻¹) compared to the rate of CO₂ use (16 μmol mg⁻¹ Chl h⁻¹) suggests that in Chlamydomonas the direct uptake of the HCO₃⁻ ion contributed about 4 times as much carbon to photosynthesis as did the CO₂. The direct transport of HCO₃⁻ was also supported by the high rate of O₂ evolution (46 μmol mg⁻¹ Chl h⁻¹) that was observed when the [CO₂] had been reduced to close to zero (Fig. 6, just before addition of carbonic anhydrase). Under these conditions the maximum rate of CO₂ supply from the spontaneous dehydra-
tion of HCO₃⁻ (calculated according to Espie et al. [6]) could only be responsible for about 29% of the observed rate of O₂ evolution, again suggesting that the transport of HCO₃⁻ ion supplied the additional carbon. Consequently, while we have directly demonstrated the active transport of CO₂ (Figs. 2, 3, and 6), our results are fully consistent with earlier publications (8, 13, 29) that suggested the active transport of both CO₂ and HCO₃⁻ in Chlamydomonas and other green algae.

In the presence of carbonic anhydrase where CO₂ and HCO₃⁻ are kept in equilibrium, it is impossible to determine the proportion of photosynthesis sustained by CO₂ transport and that sustained by HCO₃⁻ transport. In the absence of carbonic anhydrase, when the CO₂ concentration was less than 0.1 μM (Fig. 6, just before the addition of carbonic anhydrase), the maximum rate of CO₂ supply by spontaneous dehydra-
tion of HCO₃⁻ (78 nmol mL⁻¹ h⁻¹) could only support 29% of the observed rate of photosynthesis (273 nmol mL⁻¹ h⁻¹). In these circumstances, HCO₃⁻ transport was presumably supplying the remaining carbon for photosynthesis. As has been discussed previously (6), the proportion of CO₂ or HCO₃⁻ that will be used by the cells for photosynthesis will not be constant but will depend upon the availability of each species. The availability of each species will, in turn, depend upon the DIC concentration, the pH, and the presence or absence of carbonic anhydrase. The proportion of CO₂ or HCO₃⁻ used will also depend upon whether or not there is a separate transporter for each species and the affinity and maximum capacity of the transporters.

Although Palmqvist et al. (21) used a method for measuring DIC uptake that depends upon external carbonic anhydrase maintaining the CO₂/HCO₃⁻ equilibrium, they and other authors (1, 18, 20, 25, 28, 29) have questioned the essentiality of the external carbonic anhydrase to photosynthesis of low CO₂ grown Chlamydomonas and other green algae. Our results show that, indeed, the external carbonic anhydrase was not essential for a measurable rate of photosynthesis (Fig. 6, just before the addition of carbonic anhydrase) and, of course, it would not be required at all in the special case where, because of low cell density or low photosynthetic rates (29), the rate of CO₂ removal from the medium was equal to or less than the rate of spontaneous dehydra-
tion of HCO₃⁻ to CO₂. But, in our experimental conditions, when carbonic anhydrase was added, the rate of photosynthesis was more than doubled (Fig. 6, just after addition of carbonic anhydrase) and the rate of photosynthesis at the beginning of the experiments was 25% higher when carbonic anhydrase was present (Figs. 4 and 5) than when carbonic anhydrase was absent (Fig. 6). These results, then, show that while the external carbonic anhydrase is not essential for photosynthesis of low CO₂ grown Chlamydomonas, it is essential for the maintenance of max-
imum rates of photosynthesis under low DIC conditions by ensuring that both the CO₂ transporter and the HCO₃⁻ transporter are operating at their maximum capacity under the existing conditions. In the absence of external carbonic anhydrase, the CO₂ transporter could be limited by a lack of substrate so that, as Findenegg (8) stated, the external carbonic anhydrase is "an important factor in the regulation of the use of HCO₃⁻ and CO₂ for photosynthesis."
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LITERATURE CITED