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Active Transport and Accumulation of Bicarbonate by a Unicellular Cyanobacterium

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The rates of inorganic carbon accumulation and carbon fixation in light by the unicellular cyanobacterium *Coccochloris peniocystis* have been determined. Cells incubated in the light in medium containing $H^{14}CO_3^-$ were rapidly separated from the medium by centrifugation through silicone oil into a strongly basic terminating solution. Samples of these inactivated cells were assayed to determine total ^{14}C accumulation, and acid-treated samples were assayed to determine ^{14}C fixation. The rate of transport of inorganic into illuminated cells was faster than the rate of CO_2 production in the medium from HCO_3^- dehydration. This evidence for HCO_3^- transport in these cells is in agreement with our previous results based upon measurements of photosynthetic O_2 evolution. A substantial pool of inorganic carbon was built up within the cells presumably as HCO_3^- before the onset of the maximum rate of photosynthesis. Large accumulation ratios were observed, greater than 1,000 times the external HCO_3^- concentration. Accumulation did not occur in the dark and was greatly suppressed by the photosynthesis inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethyl urea and 3-chloro-carbonylcyanide phenylhydrazone. These results indicate that the accumulation of inorganic carbon in these cells involves a light-dependent active transport process.

The unicellular cyanobacterium *Coccochloris peniocystis*, like most cyanobacteria, photosynthesizes optimally at alkaline pH. We have recently shown that most of this photosynthesis is based upon exogenous HCO_3^- rather than CO_2 (11). This conclusion rests largely on the measured rate of CO_2 fixation at alkaline pH being as much as 50-fold the maximum rate of CO_2 production from the spontaneous dehydration of HCO_3^- in the external medium (11). It appears that a large HCO_3^- influx (at least 100 μeq/mg of chlorophyll per h) must take place across the cell membrane in exchange for OH^- produced within the cell (11).

Convincing evidence for the intracellular accumulation of HCO_3^- within cells of *Anabaena variabilis* has recently been presented by Badger et al. (1). After cells had been rapidly separated from the incubation solution by centrifugation through a layer of silicone fluid, inorganic carbon concentrations as much as 1,000 times the external concentration could be measured inside the cells (1). This accumulation was severely reduced by various inhibitors of energy metabolism. The inorganic carbon within the intracellular pool appeared to serve as an intermediate in photosynthesis (1).

We have used the centrifugal filtration method to demonstrate active HCO_3^- transport and accumulation by the unicellular cyanobacterium *C. peniocystis*. Accumulation ratios of more than 500 (internal concentration relative to external) were obtained, and the accumulation process was severely reduced by inhibition of energy production.

MATERIALS AND METHODS

Organism and growth conditions. All experiments were performed with *C. peniocystis* Kutz (University of Texas Culture Collection no. 1548) obtained as an axenic culture from the algal collection at Indiana University, Bloomington, Ind. Cells were cultured on air levels of CO_2 as previously described (10). Cells were harvested by centrifugation at about 15,000 × *g* for 1 min at room temperature and washed once with 50 mM potassium phosphate buffer, pH 8.0. Cell suspensions were then placed in the chamber of a Clark-type O_2 electrode (5) and allowed to reach the CO_2 compensation point as previously described (11). Samples (50 μl) of cell suspension were then removed for the assay of HCO_3^- uptake, the chamber was closed, and a small amount (10 μmol of dissolved inorganic carbon) of $NaHCO_3$ was injected to initiate O_2 evolution.

Assay of HCO_3^- uptake. Cells were rapidly separated from the bulk of the incubation medium by centrifugation through a layer of silicone fluid (7, 9). To 400-μl Eppendorf microtubes was added 100 μl of 10% methanol in 2 M NaOH (henceforth called the terminating solution) followed by 50 μl of silicone fluid (60% AR20 and 40% AR200 [vol/vol] silicone fluids; Wacker-Chemie, Munich, F.R.G.), and the two layers were compacted in the tubes by a brief centrifugation. The silicone fluid was maintained at 28°C until used.

The 50 μl of cell suspension (20 μg of chlorophyll per ml) from the electrode chamber at CO_2 compensation point was layered on top of the compacted layers placed in the head of an Eppendorf microcentrifuge (with the lid removed), and the incubation layer was illuminated by light from a slide projector (about 3×10^4 μW/cm^2 incident upon surface). The cell suspension was illuminated for 2 min before the injection into it of 10 μl of buffer containing $NaH^{14}CO_3$ (1 to 2 μCi/μmol of C). Tests with similar injection of a dye demonstrated that good mixing with the cell suspension was achieved. The light-mediated uptake of inorganic carbon was terminated by spinning the cells out of the upper aqueous incubation layer, through the silicone fluid, and into the terminating solution. Cell recovery averaged 60% after a 7-s spin at full speed (about 15,000 × *g*), and half of these recovered cells reached the terminating solution by 3 s. Longer times were needed for more quantitative recoveries, and the 7-s spin was taken as a compromise between better recoveries and the ambiguities introduced into kinetic studies by longer spins.

After termination of uptake the tubes were quickly frozen in a dry ice-methanol mixture and were stored frozen for no more than 2 h before analysis. The bottoms of the incubation tubes were cut off while still frozen at the interface of the terminating solution with the silicone fluid layer. The terminating solution was allowed to thaw and removed with a Hamilton syringe; a further 100 μl of 2 M NaOH was used as a rinse. A 50-μl sample of the resulting 200-μl volume was assayed for ^{14}C by scintillation spectrometry in 10 ml of Bray's solution containing 500 μl of ethanolamine. The remaining 150 μl of basic solution was acidified by the addition of 200 μl of 2 M perchloric acid. A 50-μl sample was removed and placed in an empty scintillation counting vial, and acid-labile ^{14}C was removed in a CO_2 stream. ACS scintillation cocktail (10 μl; Amersham/Searle) was then added, and the ^{14}C content was assayed by liquid scintillation spectrometry with a model Mark II spectrometer (Nuclear-Chicago Corp.) in the channels-ratios mode.

Expression of results. In each experiment the total fluid volume of the recovered cells was determined by the addition of ^3H_2O to the incubation layer, whereas the volume of the contaminating extracellular fluid was determined with [^{14}C]inulin or [^{14}C]sorbitol. The magnitude of cell recovery was determined by chlorophyll measurement of cells centrifuged through the silicone fluid used in the experiments, but into a 6% sucrose solution rather than the usual methanolic NaOH. From these measurements, obtained in each experiment, it was possible to calculate rates in terms of micromoles of C per milligram of chlorophyll.

In all figures, each data point is the mean of three individual determinations.

RESULTS

The uptake of ^{14}C from $NaH^{14}CO_3$ was determined by the silicone-fluid centrifugation method, in which enzymatic reactions are stopped by spinning the cells into a strongly basic medium. The ^{14}C activity of the inactivated cells is a measure both of the ^{14}C fixed into organic compounds and ^{14}C-labeled inorganic carbon remaining unfixed. The ^{14}C activity remaining after acidification of the cells gives a measure of ^{14}C fixed, and subtraction of this amount from the total ^{14}C incorporated yields the activity of the ^{14}C-labeled inorganic carbon. A typical time course of incorporation into these fractions in illuminated cells is shown in Fig. 1. For about 30 s the amount of carbon accumulated by the cells but remaining unfixed exceeds the amount fixed into acid-stable products (Fig. 1). The relationship between these two components is indicative of a precursor-product relationship, with acid-labile carbon eventually becoming fixed into acid-stable forms by photosynthesis. Uptake of carbon is so rapid that even substantial errors in the determination of the volume of contaminating medium in the algal pellet would have little effect upon estimations of HCO_3^- accumulation. Thus, even after incubations of illuminated cells in $H^{14}CO_3^-$ for periods as short as 10 s, the extracellular $H^{14}CO_3^-$ concentration amounted to only 2.6% of the estimated intracellular acid-labile carbon (Fig. 1).

To determine whether the acid-labile ^{14}C in the cells resulted from decarboxylation of an

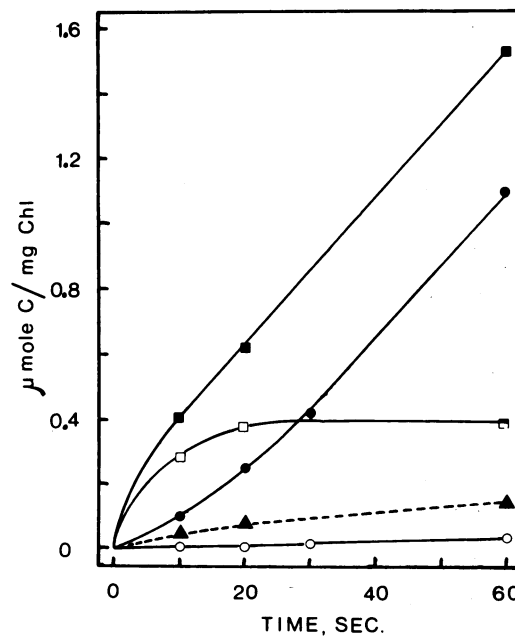

FIG. 1. *Inorganic carbon accumulation by illuminated C. peniocystis. The initial inorganic carbon concentration was 42 μM, pH 8.0 Symbols:* ■, *total carbon taken up;* ●, *carbon photoassimilated into acid-stable products;* □, *inorganic, unassimilated carbon within the cells;* ○, *ether-extractable carbon (lipids and keto acid phenylhydrazones); and* ▲, *calculated uptake supportable solely by CO_2 transport.*

unstable keto acid product of photoassimilation, experiments were carried out with 2% phenylhydrazine in 2 M perchloric acid as a terminating solution, rather than the usual methanolic NaOH. No significant change in the proportions of the various forms of carbon within the cells was observed (Fig. 1).

Unfixed inorganic carbon (mainly HCO_3^-) in the cells reached an estimated internal concentration of 2.9 mM. This concentration represents a 200-fold accumulation with respect to the external inorganic carbon (mostly HCO_3^-) concentration.

For a known pH and total dissolved inorganic carbon (DIC) concentration it is possible to calculate a maximum rate of CO_2 formation from spontaneous HCO_3^- dehydration in the medium (11). In a closed system the spontaneous dehydration of HCO_3^- at alkaline pH will redress the loss of CO_2 removed by photosynthesizing cells. If it is assumed that the cells transport only CO_2, and if it is assumed that CO_2 transport is so efficient that CO_2 molecules are scavenged by the cells as soon as they are produced by dehydration of HCO_3^- in the medium, the maximum CO_2 supply rate (and thus the maximum transport rate) can be estimated from the equation: $d[CO_2]/dt = ([DIC]/5{,}000\ A)(1 + 5.25 \times 10^8 [H^+])$, where $A = 1 + ([H^+]/4.45 \times 10^{-7}) + (4.69 \times 10^{-11}/[H^+])$. The calculations apply only to a closed system, but in the completely unstirred incubation layers used in the uptake assays significant entry of atmospheric CO_2 over the first 10 s or so of uptake would not be expected. In any event, the rate of CO_2 formation calculated according to Miller and Colman (11) would be a large overestimate since the cells would not be able to scavenge CO_2 molecules as rapidly as they are formed by HCO_3^- dehydration (a simplifying assumption used to derive the rate equation). For the first 10 s or so of carbon uptake (Fig. 1) the observed transport rate into the cells was 10 times the maximum rate of transport that could have been obtained if CO_2 were the only transported molecule. At higher pH values and lower DIC concentrations than those used in the uptake assays, an even greater inability of CO_2 transport alone to support the observed rate of transport would be expected.

The uptake of inorganic carbon and the formation of an intracellular pool was much reduced in the dark. Within 10 s of turning the lights off, inorganic carbon transport was reduced by about 90% (Fig. 2). Carbon fixation was reduced more severely (97%) than was formation of the inorganic carbon pool (92%). The concentration of the internal HCO_3^- pool reached a level of only 11 times (0.43 mM) the external HCO_3^- concentration in the dark compared with 200-fold or greater accumulations observed with illuminated cells.

The effect of darkness upon retention of an intracellular pool formed in the light was also studied (Fig. 3). Upon turning the lights off, the net transport of inorganic carbon ceased, and within 10 s no further incorporation of carbon into acid-stable products occurred. There was, in fact, a net efflux of inorganic carbon from the cells at the expense of the intracellular pool (Fig. 3).

3-(3,4-Dichlorophenyl)-1,1-dimethyl urea (DCMU), an inhibitor of noncyclic photophosphorylation, significantly reduced total inorganic carbon transport, intracellular pool formation, and photoassimilation into acid-stable products (Fig. 4). The final intracellular concentration of HCO_3^- reached was lowered from 3.1

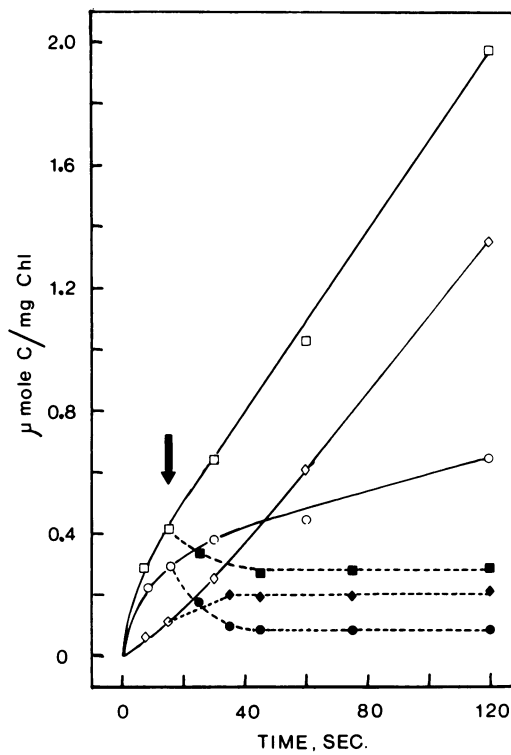

FIG. 2. *Inhibition of carbon uptake by darkness. Cells were illuminated in the centrifuge tubes for 2 min and then illuminated for 1 min (control, 0 sec) or preincubated in the dark for various lengths of time up to 2 min. At the end of these preincubation times all cells were assayed for carbon uptake over a standard 10-s period, either in the light (control) or dark. The initial DIC concentration was 41 μM at pH 8.0. Symbols: ●, acid-labile carbon; ■, acid-stable carbon.*

mM in the control to 1.8 mM in the presence of 10 μM DCMU. In the former case, the accumulation ratio was about 360, whereas it was only 84 in the presence of DCMU (a 77% reduction). Light-dependent O_2 evolution was completely abolished by 10 μM DCMU (data not shown), but assimilation of ^{14}C into acid-stable products was not (Fig. 4). Presumably, appreciable fixation can occur by a reaction catalyzed by enzymes such as phosphoenol pyruvate carboxylase (4).

The uncoupler 3-chloro-carbonylcyanide phenylhydrazone (CCCP) at 10 μM also caused significant inhibition of total uptake of inorganic carbon and pool formation (Fig. 5). In the absence of CCCP there was an intracellular accumulation of HCO_3^- to a level 228 times that in the external medium, whereas in the presence of CCCP this accumulation was reduced 68-fold, a 70 % reduction in accumulation.

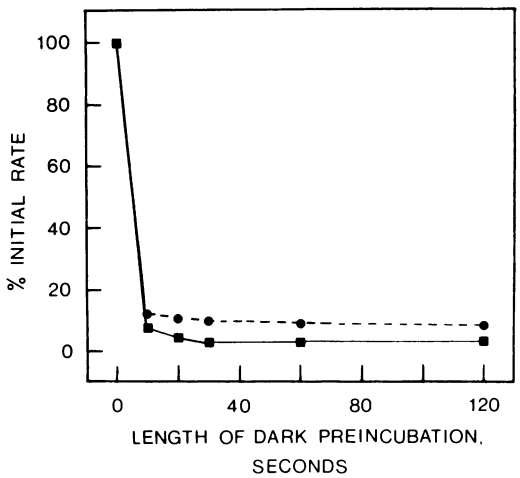

FIG. 3. *Effect of darkness on retention of accumulated HCO_3^-. The DIC concentration was 42 μM at pH. 8.0. Total accumulated carbon, in light (□) and after transfer to dark (■); fixed carbon, in light (◇) and after transfer to dark (◆); inorganic unassimilated carbon, in light (○) and after transfer to dark (●).*

In one instance, the uptake of inorganic carbon was followed over a longer than usual time (Fig. 6). By 4 min, HCO_3^- had accumulated in the intracellular pool to a concentration more than 1,000 times the external concentration (4.8 mM versus 3 μM). However, it must be emphasized that there is some uncertainty as to the true magnitude of the intracellular HCO_3^- concentration and the accumulation ratio. This is due to the difficulty of measuring the true specific activity of the inorganic carbon in the medium after long incubation periods. There is a good indication that, after the longer incubation periods, photoassimilation of inorganic carbon proceeds at the expense of HCO_3^- in the intracellular pool (Fig. 6).

DISCUSSION

The centrifugal filtration technique has been used to measure the extent of inorganic carbon accumulation by *C. peniocystis* (Fig. 1). The rate of accumulation was much faster than that supported solely by the movement of CO_2 across the cell membrane (Fig. 1). That is, the maximum CO_2 supply rate that could result from spontaneous dehydration of HCO_3^- in the external medium was much too low to account for the observed rate of inorganic carbon accumulation within the cells. No evidence for extracellular

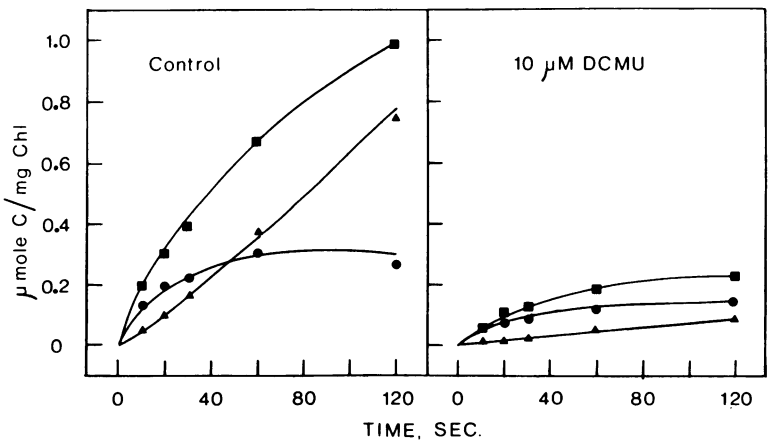

FIG. 4. *Effect of 10 μM DCMU on carbon transport and accumulation. DIC concentration was 28 μM at pH 8.2. DCMU dissolved in 1% ethanol was added to a final concentration of 10 μM at the beginning of the 2-min preillumination period. To the control cell suspension was added 1% ethanol. Symbols: ■, total carbon uptake; ▲, photoassimilated carbon; ●, inorganic, unassimilated carbon within the cells.*

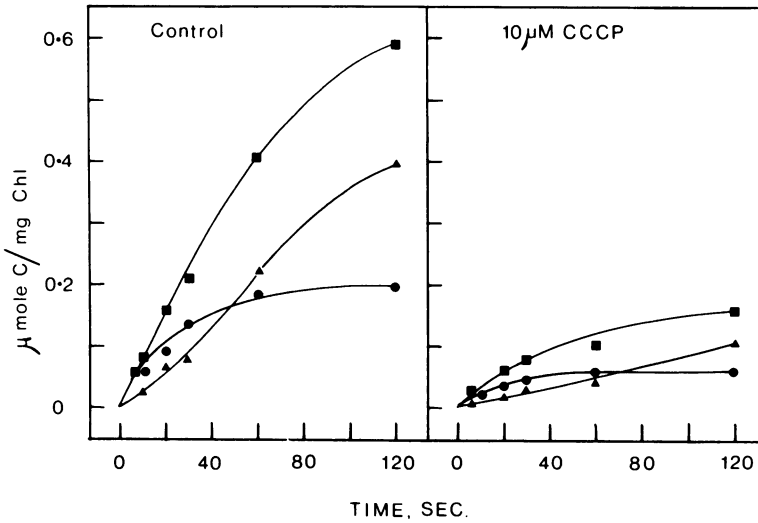

FIG. 5. *Effect of 10 μM CCCP in carbon transport and accumulation. DIC concentration was 10 μM at pH 8.0. Other conditions were as for Fig. 4. Symbols: ■, total carbon uptake; ▲, photoassimilated carbon; ●, inorganic, unassimilated carbon within the cells.*

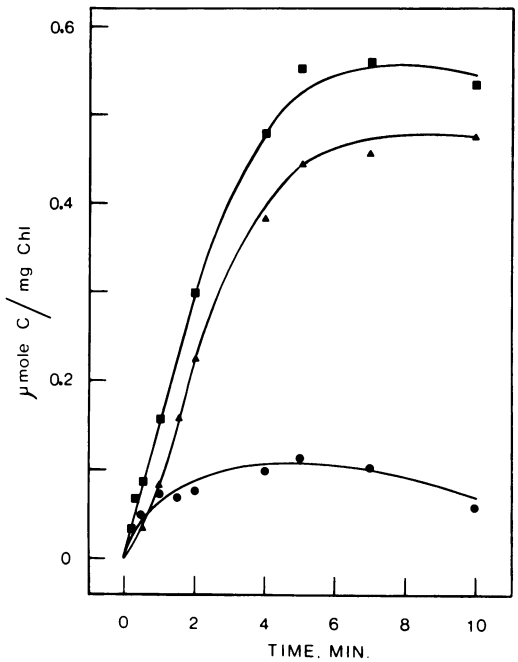

FIG. 6. *Long-term incubation. DIC concentration was 10 μM at pH 8.0. Other conditions were as described for Fig. 4. Symbols: ■, total carbon uptake; ▲, photoassimilated carbon; ●, inorganic, unassimilated carbon within the cells.*

carbonic anhydrase production by *C. peniocystis* has been found (11). Thus, the results shown in Fig. 1 demonstrate that inorganic carbon accumulation must be mediated mainly by HCO_3^-, and not CO_2, transport. We have already shown that photosynthetic carbon fixation depends upon HCO_3^- uptake from the medium (11).

The accumulation ratios for inorganic carbon within the cells were as high as 1,000 (Fig. 6). Similar ratios have been obtained with *A. variabilis* (1). Consideration of the accumulation ratios leads to the conclusion that HCO_3^- moves into the cells against its electrochemical gradient. In the present study we had difficulty obtaining consistent results for the intracellular pH using ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione, but subsequent refinements in the technique (J. R. Coleman and B. Colman, Plant Physiol., in press) yield a similar alkaline pH value (ca. 7.9) obtained for bulk cytoplasm in the light under the conditions used in the present study. At this pH almost all of the accumulated inorganic carbon will be HCO_3^-, as it is at the external pH of 8.0. The accumulation ratio can thus be considered as the ratio of intracellular to extracellular HCO_3^- concentration. If HCO_3^- were passively distributed across the cell membrane, an inwardly directed, positive potential of about +180 mV would be required to account for the observed 1,000-fold HCO_3^- accumulation shown in Fig. 6. Even if the estimate of the free intracellular HCO_3^- was reduced to 10% of the observed accumulation value, a potential of +60 mV (inside positive) would still be required to account for passive HCO_3^- accumulation. Much of the accumulated HCO_3^- was rapidly lost from cells placed in the dark (Fig. 2), suggesting that considerably more than 10% of the accumulated HCO_3^- was in fact readily

diffusible within the cells. The membrane potential of *C. peniocystis* has not yet been determined, but in the closely related *Anacystis nidulans* (13) a potential of −107 mV in the dark has been determined (12). No positive membrane potentials have been determined in any procaryotes (8). It is much more likely that HCO_3^- is moved into the cells of *C. peniocystis* against its electrochemical gradient than by passive movement into the cells in response to a large, positive membrane potential difference. The severe inhibitory effect of CCCP and DCMU upon HCO_3^- accumulation is consistent with this hypothesis (Fig. 4 and 5).

A minimum estimate of the HCO_3^- influx at a total external inorganic carbon concentration of 42 μM is about 470 μeq/mg of chlorophyll per h (Fig. 1). We have not yet attempted a detailed kinetic analysis of HCO_3^- influx into *C. peniocystis* because a number of quite formidable technical difficulties prevent a fully quantitative interpretation at this time. These problems have not always been specifically mentioned by other workers (1). Some of the problems are as follows.

(i) To avoid ambiguity as to the form of inorganic carbon transported (i.e., HCO_3^- or CO_2), low external inorganic carbon concentrations must be used. At higher inorganic carbon concentrations the rate of HCO_3^- dehydration could provide enough CO_2 to account for the inorganic carbon accumulation. Unfortunately, it is very difficult to determine the true specific activity of the inorganic carbon at these low concentrations. Although care was always taken to avoid undue entrance of atmospheric CO_2 during preparation of the incubation layer, the specific activity was probably lower than the nominal value. The HCO_3^- influxes quoted in this paper are thus probably underestimated. Although a sensitive gas-chromatographic technique has been developed in this laboratory for measuring dissolved inorganic carbon (2), it will be difficult to measure the true inorganic carbon concentration in the small (50-μl) volumes of cell suspension at the actual time of uptake.

(ii) The size of the nonradioactive inorganic pool within the cells at the beginning of HCO_3^- transport is unknown. Certainly, there is no reason to suspect that some of the $H^{14}CO_3^-$ influx does not represent exchange for intracellular HCO_3^- rather than net uptake by $H^{14}CO_3^-$-OH^- exchange. This problem does not seem to have been adequately considered in recent reports (1, 6). It is possible that a good estimate of the HCO_3^--OH^- exchange can be obtained by measurement of the OH^- efflux occurring within the first 10 s of HCO_3^- addition.

(iii) The accumulation of radioactive inorganic carbon by *C. peniocystis* is so rapid (Fig. 1) that even by the first feasible sampling time (ca. 5 s) considerable back-flux of $^{14}CO_2$ and $H^{14}CO_3^-$ from the cells is probable. This problem has been considered by Badger et al. (1), and it will result in an underestimation of the actual HCO_3^- influx. Rapid measurement of HCO_3^--OH^- may partially overcome this problem.

(iv) The centrifugal filtration technique necessitates the use of an unstirred incubation layer. The rates of HCO_3^- influx are so rapid that large OH^- and HCO_3^- gradients will rapidly be created, resulting in reduced fluxes. It is difficult to overcome this inherent problem. Once again, a rapid measurement of OH^- fluxes in well-stirred cell suspensions might be a valuable alternative.

Although the actual size of the net HCO_3^- uptake is in doubt, it is clear that HCO_3^- accumulation occurs, probably by an energy-requiring process. The ability of *C. peniocystis* and other cyanobacteria and microalgae to scavenge and accumulate inorganic carbon when it is present at low concentrations seems to account for the low CO_2 compensation points and low photorespiratory rates observed in these species (2, 3). It must be remembered, however, that in both the C-4 photosynthetic metabolism of higher plants and in the efficient carbon accumulation of cyanobacteria and microalgae a considerable energy cost is involved. Further investigation will involve attempts to accurately determine the magnitude of the HCO_3^- uptake and the associated energy requirement.

ACKNOWLEDGMENT

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