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Active Transport of CO_2 by the Cyanobacterium *Synechococcus* UTEX 625[1]

MEASUREMENT BY MASS SPECTROMETRY

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ABSTRACT

Mass spectrometry has been used to confirm the presence of an active transport system for CO_2 in *Synechococcus* UTEX 625. Cells were incubated at pH 8.0 in 100 micromolar $KHCO_3$ in the absence of Na^+ (to prevent HCO_3^- transport). Upon illumination the cells rapidly removed almost all the free CO_2 from the medium. Addition of carbonic anhydrase revealed that the CO_2 depletion resulted from a selective uptake of CO_2, rather than a total uptake of all inorganic carbon species. CO_2 transport stopped rapidly (<3 seconds) when the light was turned off. Iodoacetamide (3.3 millimolar) completely inhibited CO_2 fixation but had little effect on CO_2 transport. In iodoacetamide poisoned cells, transport of CO_2 occurred against a concentration gradient of about 18,000 to 1. Transport of CO_2 was completely inhibited by 10 micromolar diethylstilbestrol, a membrane-bound ATPase inhibitor. Studies with DCMU and PSI light indicated that CO_2 transport was driven by ATP produced by cyclic or pseudocyclic photophosphorylation. Low concentrations of Na^+ (<100 microequivalents per liter), but not of K^+, stimulated CO_2 transport as much as 2.4-fold. Unlike Na^+-dependent HCO_3^- transport, the transport of CO_2 was not inhibited by high concentrations (30 milliequivalents per liter) of Li^+. During illumination, the CO_2 concentration in the medium remained far below its equilibrium value for periods up to 15 minutes. This could only happen if CO_2 transport was continuously occurring at a rapid rate, since the continuing dehydration of HCO_3^- to CO_2 would rapidly raise the CO_2 concentration to its equilibrium value if transport ceased. Measurement of the rate of dissolved inorganic carbon accumulation under these conditions indicated that at least part of the continuing CO_2 transport was balanced by HCO_3^- efflux.

Photosynthesis by cyanobacteria can occur when the CO_2 concentration in the extracellular medium is so low that CO_2 fixation via Rubisco[2] could not occur were it not for the presence of 'CO_2-concentrating' mechanisms (1, 2, 9, 13, 16, 19, 21, 25, 28). With a combination of alkaline pH, low DIC concentration and high cell density, photosynthesis by cyanobacteria is sustained mainly by the uptake of HCO_3^-, rather than CO_2, from the medium (2, 8, 9, 16, 19, 20). However, Badger and Andrews (2), using a mass spectrometer, provided strong evidence that a marine species of *Synechococcus* was also capable of removing CO_2 from the medium by an active transport process. This was most evident upon the illumination of darkened cells, when CO_2 was taken up so rapidly by the cells that the CO_2 concentration in the extracellular medium dropped almost to zero, in spite of the continuous reformation of CO_2 in the medium due to the dehydration of HCO_3^- (16).

While the mass spectrometric data of Badger and Andrews (2) are the most direct evidence yet presented, other approaches have yielded data that are fully consistent with the concept of active CO_2 transport by cyanobacteria. At pH 8.0, in the absence of added CA, the rate of CO_2 hydration to HCO_3^- is low enough ($t_{1/2} \simeq 5$ s at 30°C) that cells can be provided, for a short period of time, with CO_2 at concentrations that are considerably in excess of the rather low equilibrium concentrations (only about 2% of the total DIC concentration at pH 8.0, 30°C, and low ionic strength). A number of species of cyanobacteria have been shown to be capable of accumulating DIC against considerable concentration gradients under these conditions of high, non-equilibrium CO_2 to HCO_3^- ratios (1, 2, 9, 18, 29). For a given DIC concentration, the rate of DIC accumulation was faster under the nonequilibrium conditions (high CO_2/HCO_3^-) than under equilibrium conditions (high HCO_3^-/CO_2), thus indicating a lower K_m for CO_2 transport than for HCO_3^- transport (1, 2, 9, 29).

Miller and Canvin (17) provided further evidence for a CO_2-transport capacity, distinct from the HCO_3^- transport capacity, when they made use of the observation that HCO_3^- transport in rapidly growing cells of *Synechococcus* UTEX 625 is inhibited by the absence of Na^+ in the extracellular medium (8, 17, 22). Cells that were incubated in the absence of Na^+ were stimulated to accumulate normal levels of intracellular DIC by the addition of CA (17). It was postulated that, in the absence of the CA, the rate of supply of CO_2 to the CO_2-transport system was limited by the rate of HCO_3^- dehydration to CO_2 in the extracellular medium. The DIC transport occurring in the presence of CA was not inhibited by the addition of Li^+, whereas the Na^+-dependent process of HCO_3^- transport was strongly inhibited (17).

In the present study we have used a mass spectrometer to further describe the process of active CO_2 transport by the cyanobacterium *Synechococcus* UTEX 625.

MATERIALS AND METHODS

Organism and Growth Conditions. *Synechococcus* UTEX 625 (*S. leopoliensis*, University of Texas Culture Collection), also known as *Anacystis nidulans* (27) was grown with air bubbling

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[2] Abbreviations: Rubisco, D-ribulose 1,5-bisphosphate carboxylase/oxygenase; BTP, 1,3-bis(tris[hydroxymethyl]methylamino)-propane; CA, carbonic anhydrase; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DES, diethylstilbestrol; DIC, dissolved inorganic carbon (CO_2 + HCO_3^- + CO_3^{2-}); $K_{1/2}$, substrate concentration required to give one-half maximum rate; $[CO_2]_i$, intracellular concentration.

in a modified Allen's medium (8, 21) to a yield of 7 to 10 μg Chl·ml^{-1}. Just before use, cells were washed (three times) and resuspended in 25.0 mM BTP/23.5 mM HCl buffer (pH 8.0). This buffer contains only 10 to 20 μM DIC (or 0.15 – 0.30 μM CO_2) when it is kept under N_2 in a serum-stoppered flask (21).

$^{14}CO_2$ Uptake. The accumulation of ^{14}C-DIC was measured by rapidly separating cells from the incubation medium by centrifugal filtration through silicone fluid (6, 13). Samples (10 μl) of cell suspension were layered over 75 μl silicone fluid which, in turn, rested on 100 μl of 2 M KOH/10% methanol in a 400 μl microcentrifuge tube. The cell samples were then covered with 75 μl degassed mineral oil (USP heavy) to reduce the invasion of atmospheric CO_2. All operations were carried out under a stream of N_2 to further reduce CO_2 contamination of the cell sample. The silicone fluid used was a mixture of Wacker-Chimie (Munich, FRG) AR20 and AR200 in a 0.625 to 1.0 ratio. The viscosity of these silicone fluids makes accurate measurement difficult and in some cases small amounts of either AR20 or AR200 had to be added to the mixture to give good recovery (>85%) of the unicellular *Synechococcus* into the basic terminating solution or to prevent the inversion of the layers during centrifugation. One tube at a time was placed in a Beckman microfuge and illuminated from the side with 300 W Sylvania flood-lamps yielding an incident photon flux density (400–700 nm) of about 350 μE/m^2·s, measured with a Li Cor model LI-185 light meter. The transmittance of this light through the polypropylene walls of the tubes was about 70%. With the aid of a water filter between the microcentrifuge tubes and the lamp and a fan blowing into the microcentrifuge, the temperature was maintained between 27 and 30°C. $^{14}CO_2$ solutions were generated in sealed microcentrifuge tubes by the addition of $K_2{}^{14}CO_3$ solution (0.7–1.2 μCi/μmol) to 10 mM phthalic acid at pH 4.0. Before each series of uptake measurements the $^{14}CO_2$ content of the stock solution was determined by liquid scintillation spectrometry and the volume of the stock solution needed to yield the desired CO_2 concentration in the incubation suspensions was calculated. The uptake of $^{14}CO_2$ was initiated by the injection of the required volume of the $^{14}CO_2$ solution (5–8 μl, with H_2O to give a total volume of 10 μl). Uptake was terminated by centrifuging the cells through the silicone fluid. The incubation layer became noncolored to the eye only after about 9 s centrifugation. The basic terminating solution was assayed for unmetabolized and metabolized ^{14}C-DIC as previously described (6).

Mass Spectrometry. The uptake of $^{12}CO_2$ or $^{13}CO_2$ by cells was monitored by continuous measurement of the CO_2 concentration in the extracellular medium by mass spectrometry using an aqueous inlet system (2, 12). A VG Gas Analysis (Middlewich, England), model MM 14-80 SC, magnetic sector mass spectrometer was used. Cells suspensions (5 ml), washed three times in BTP/HCl buffer, were incubated in a glass cuvette (20 mm diameter) that contained a magnetic stirrer-bar and was thermostated at 30°C. The chamber was closed with a Plexiglas plug so as to leave no headspace. The plug contained a capillary bore for injection purposes. The inlet capillary to the mass spectrometer was separated from the cell suspension by a thin dimethyl silicone rubber membrane supported by a metal grid. The response time for CO_2 measurement was determined by the injection of K_2CO_3 solutions into pH 8.0 buffer in the presence of CA (25 μg/ml). Response time (63% full response) was about 2 s. Calibration for CO_2 was achieved by the injection of small volume of 100 mM K_2CO_3 and calculation of the equilibrium CO_2 concentration for pH 8.0 at 30°C according to Buch (5). At the end of each run, 25 μl of 2 N KOH was injected into the cell suspension to raise the pH above 11 so that a measurement at essentially 0 μM CO_2 could be obtained. The leak rate of CO_2 across the inlet membrane to the mass spectrometer was low, <0.2%/min, yet the sensitivity of measurement was high (signal to noise ratio at 0.1 μM CO_2 was about 10 to 1). The cell suspensions usually (5–8 μg Chl/ml) were continuously stirred during the measurements. In most experiments incident photon flux density was about 600 μE/m^2·s PAR and was provided by a quartz-halogen lamp. Alternatively, incident light enriched in wavelengths absorbed by PSII was obtained using a 627 nm (half-bandwidth 22 nm) Balzers interference filter. Light enriched in PSI wavelengths was obtained using a 665 nm sharp cut-on filter (Oriel) in combination with a 672 nm interference filter (Balzers) to yield light having a peak wavelength of 677 nm and a half-bandwidth of 25 nm. The photon flux density incident upon the cuvette was adjusted by means of neutral density filters to be 15 μE/m^2·s in both cases. The mass spectrometer was also used to monitor photosynthetic O_2 evolution. Calibration was obtained using H_2O_2 and catalase. catalase.

Estimation of $[CO_2]_i$. In the past we have used the value of the carbonic acid dissociation constant at infinite dilution to calculate $[CO_2]_i$ (19). In this study we have used the equation derived by Yokota and Kitaoka (31) to calculate the pK_1 for carbonic acid for a more realistic cytoplasmic ionic strength of 0.2 M and buffer ionic strength of 25 mM. The respective pK_1 values are 6.04 and 6.21, which compare to an infinite dilution pK_1 of 6.33 at 30°C (11). Using these pK_1 values produces $[CO_2]_i$ values that are 46% lower than those calculated using the pK_1 relevant to infinite dilution. Usually, however, it is the $[CO_2]_i/[CO_2]_o$ ratio that is of interest and in this case the ratio based on pK_1 values relevant to the ionic strength of the cytoplasm and external buffer are 31% lower than those based on the pK_1 relevant to infinite dilution. These calculations are based on the assumption that the intracellular pH in the light is 7.5 (4, 6). While calculations of $[CO_2]_i$ should certainly be made using pK_1 values relevant to the cytoplasmic ionic strength (31) it should be borne in mind that an uncertainty of only 0.1 pH in the value for the intracellular pH produces an uncertainty in the $[CO_2]_i$ of 20%. An intracellular volume of 75 μl/mg Chl was used when calculating $[DIC]_i$ and $[CO_2]_i$ (GS Espie, AG Miller, DT Canvin, unpublished data).

Chemicals. Carbonic anhydrase, BTP, CCCP, and DES were obtained from Sigma Chemical Co. The $K_2{}^{13}CO_2$ (99 atom % ^{13}C) was obtained from MSD Isotopes, Montreal, Canada. The $K_2{}^{14}CO_3$ was synthesized from $Ba^{14}CO_3$ (Atomic Energy of Canada Ltd., Kanata, Canada) by acid release of $^{14}CO_2$ with subsequent trapping in KOH.

RESULTS

Uptake of $^{14}CO_2$. Very substantial uptake of ^{14}C-DIC occurred when cells incubated in the light were provided with $^{14}CO_2$ dissolved in 10 mM phthalic acid at pH 4.0 (Fig. 1). The pH of the cell suspension remained above pH 7.9 upon the addition of the small volume of acidic solution. The percentage of the $^{14}CO_2$ taken up by illuminated cells was about 50% of the $^{14}CO_2$ provided (Fig. 1). As found by Badger and Andrews (2), but not by Volokita *et al.* (29) or Abe *et al.* (1), uptake was essentially complete by the first sampling time (Fig. 1). It must be noted that the sampling times given in Figure 1 are only nominal for they do not include the approximately 9 s required for most of the cells to be completely spun out of the incubation layer containing the $^{14}CO_2$. Uptake of $^{14}CO_2$ was substantially reduced by 10 μM DCMU as well as darkness (Fig. 1). The addition of CA to the incubation medium greatly reduced the uptake of ^{14}C-DIC following a $^{14}CO_2$ pulse (Fig. 1), thus indicating that most of the uptake occurring in the absence of CA was, in fact, as $^{14}CO_2$ and not $H^{14}CO_3^-$. Little fixation of the transported ^{14}C took place within the short time period of these experiments (about 5% fixed after 15 s) and thus most (95%) of the transported ^{14}C remained in the cells as $^{14}CO_2$ and $H^{14}CO_3^-$. If assumptions regarding the intracellular pH in the light and pK_1 for carbonic acid are made ("Materials and Methods"), then the intracellular

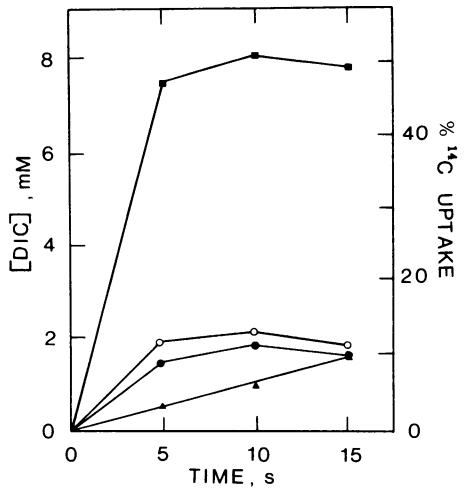

FIG. 1. Measurement of $^{14}CO_2$ uptake. The silicone fluid filtration method was used to measure the $^{14}CO_2$ uptake ("Materials and Methods") following the addition of 10 μM $^{14}CO_2$. Uptake was measured in the light (■), in the light in the presence of 25 μg CA/ml (▲), or 10 μM DCMU (○) and in the dark (●). The [DIC]$_i$ (= intracellular [DIC]) is based upon the specific activity of the added $^{14}CO_2$ in all cases. In the presence of CA the specific activity for the total [DIC] would be used if HCO_3^- transport were also occurring but since the cells were incubated in the absence of 25 mM NaCl the rate of HCO_3^- transport was low (17). Due to contaminant DIC (15 μM) the specific activity for the total [DIC] would be about two-thirds that of the $^{14}CO_2$ specific activity.

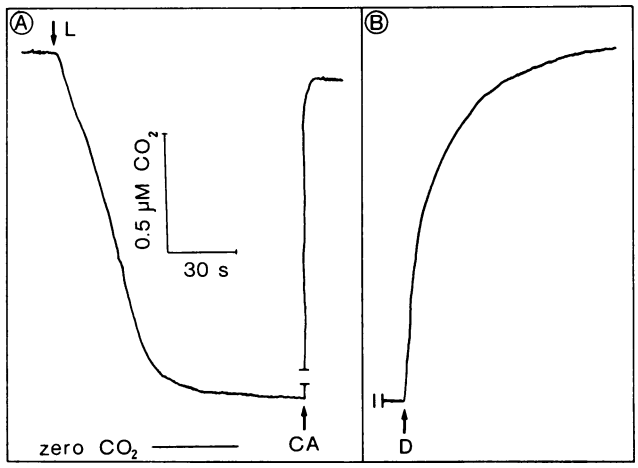

FIG. 2. Measurement of CO_2 transport by mass spectrometry. A, Cells were incubated in the dark for several minutes with 100 μM $KHCO_3$ at pH 8.0 ([CO_2] = 1.6 μM) and then CO_2 transport was initiated by turning on the lights (L). CA was subsequently added to a final concentration of 25 μg/ml. The addition of CA, even in the absence of cells, results in a 17.6% increase in the mass 44 signal, presumably due to relief of unstirred layer effects near the capillary inlet membrane (10, 12). This enhancement has been subtracted from the actual chart recorder tracing. B, The effect of darkness (D) upon CO_2 uptake, following a period of illumination, was monitored in a separate cell suspension in the absence of CA. The [Chl] was 7.5 μg/ml.

CO_2 concentration in the illuminated cells after 15 s can be estimated as 264 μM. The extracellular CO_2 concentration at this time is not known accurately, but will be lower that the initial 10 μM because of uptake by the cells and because of the conversion of CO_2 to HCO_3^- in the extracellular medium. We estimate the $[CO_2]_o$ to be about 1 μM after 15 s. This gives a $[CO_2]_i/[CO_2]_o$ of about 264 to 1.

Mass Spectrometry. Upon illumination of the cells that had been incubated in the dark for several minutes with 100 μM $KHCO_3$ at pH 8.0 ([CO_2] = 1.6 μM) there was a rapid and substantial drop in the extracellular CO_2 concentration (Fig. 2). This low CO_2 concentration was maintained for at least 10 min (the longest time examined, data not shown). The addition of CA caused a rapid return to the equilibrium CO_2 concentration (Fig. 2). The mass spectrometer measures only dissolved CO_2 concentration, since only CO_2 is volatile across the silicon rubber membrane that covers the end of the inlet capillary. Thus, in the absence of CA, a drop in the extracellular CO_2 concentration could be due either to the selective removal of CO_2 by the cells at a rate sufficient to maintain the $HCO_3^- \rightleftharpoons CO_2$ conversion out of equilibrium or could be due to the total removal of CO_2 plus HCO_3^- from the medium. The rapid restoration of a close to equilibrium CO_2 concentration upon the addition of CA argues very strongly for the former option—the selective uptake of CO_2 at a rapid rate by the cells (Fig. 2). The observation that the extracellular CO_2 concentration remained low, even though a substantial concentration of HCO_3^- remained, means that a substantial rate of CO_2 transport was being maintained (Fig. 2). Obviously, without ongoing CO_2 transport the dehydration of HCO_3^- remaining in the medium would soon raise the extracellular CO_2 concentration to its equilibrium value. This, of course, is what happens when CA is added, resulting in a rate of HCO_3^- to CO_2 conversion that exceeds the rate of CO_2 transport (Fig. 2) or when CO_2 transport is inhibited by dark (Fig. 2). The rate of ongoing CO_2 transport can be calculated from the equation:

$$Jco_2 = k_d [HCO_3^-]_t - 62\, k_d [CO_2]_t \quad (1)$$

where Jco_2 is the net influx rate, in nmol $CO_2 \cdot ml^{-1} \cdot s^{-1}$, k_d is the overall rate constant for HCO_3^- dehydration at pH 8.0, and 30° ($0.87 \times 10^{-3} \cdot s^{-1}$) and $[HCO_3^-]_t$ and $[CO_2]_t$ are the concentrations at time t. The value for k_d was obtained under the actual conditions used for CO_2 uptake by the cells, by monitoring the time course of CO_2 production after the addition of a reasonably high (1 mM) concentration of K_2CO_3. The coefficient 62 is the ratio of the equilibrium $[HCO_3^-]$ to the equilibrium $[CO_2]$, calculated for pH 8.0, 30°C, and an ionic strength of 0.025 M. For short periods after illumination (about 60 s or so) the $[HCO_3^-]$ remains quite constant if cells are incubated in the absence of the millimolar concentrations of Na^+ required for HCO_3^- transport by these cells (8, 17). In the absence of HCO_3^- transport, the total [DIC] drops quite slowly because the rate of DIC uptake is limited by the rate at which HCO_3^- is dehydrated in the medium to produce CO_2 for uptake (8, 16, 20). The addition of CA rapidly reestablishes the equilibrium between HCO_3^- and CO_2, and the CO_2 signal from the mass spectrometer is now in direct proportion to the [DIC] in the medium. As expected, the addition of CA after only a short period of illumination reveals that the total amount of DIC taken up by the cells was low, even though the [CO_2] dropped almost to zero (Fig. 2).

Other than darkness (Fig. 2), CO_2 uptake was most readily inhibited by low concentrations of DES (Fig. 3A) which is an inhibitor of various membrane-bound ATPases (15). The uncoupler CCCP, at rather high concentrations, inhibited CO_2 uptake (Fig. 3B) as did DCMU but only at concentrations substantially greater than those needed to inhibit O_2 evolution (Fig. 4). When cells were illuminated with light (subsaturating at 15 $\mu E/m^2 \cdot s$) enriched in wavelengths absorbed more by PSI than PSII O_2 evolution was inhibited by about 54% while CO_2 uptake was inhibited by only about 6% (data not shown).

Effect of Na^+, K^+, and Li^+. We have previously shown that Li^+ inhibited the Na^+-dependent transport of HCO_3^- but did not inhibit the CA-dependent transport of DIC (presumably as CO_2) (8, 17). We have also shown that the uptake of CO_2 by cells grown on 5% CO_2 was stimulated by the addition of low

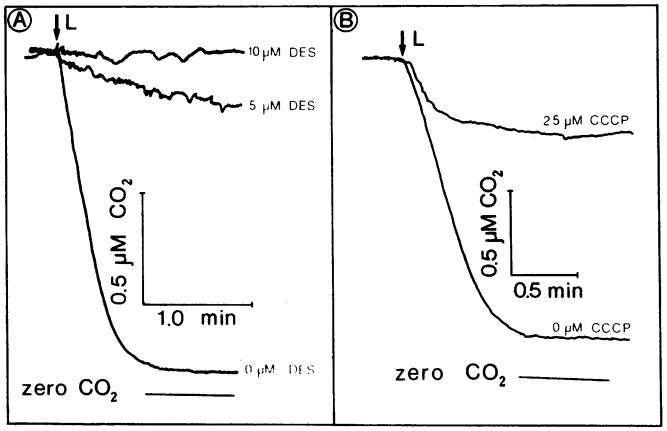

FIG. 3. Inhibition of CO_2 uptake by DES and CCCP. Cells were incubated in the dark for several minutes, in the absence or presence of inhibitor, with 100 μM $KHCO_3$ at pH 8.0 and then CO_2 uptake was initiated by turning on the lights (L). A, In the absence or presence of DES; B, in the absence or presence of CCCP. The [Chl] was 8.7 and 9.6 μg/ml, respectively.

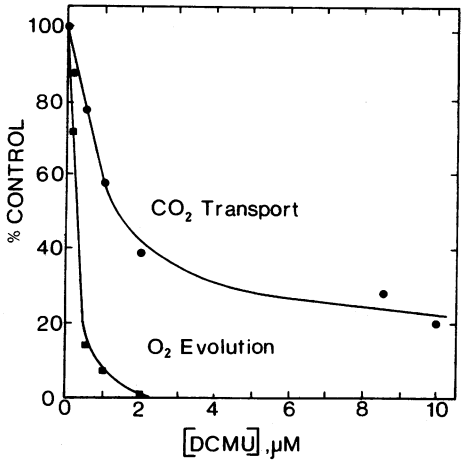

FIG. 4. Effect of DCMU concentration upon photosynthetic O_2 evolution and CO_2 transport. Steady state rates of O_2 evolution (■) were measured after successive additions of DCMU to yield increasingly higher concentrations. CO_2 transport (●) was measured by turning on the lights after DCMU had been added in the dark. The rates of CO_2 transport were based on the initial, linear rate observed in the 10 s period after turning on the light. CO_2 fixation during this period was negligible. Cells (5.1 μg Chl/ml) were incubated in 100 μM $KHCO_3$ at pH 8.0.

concentrations ($K_{1/2}$ = 18 μM) of Na^+ (18). We have now investigated the effect of these cations on the CO_2 uptake by air-grown cells (Table I). The addition of low concentrations of NaCl (100 μM) or Na_2SO_4 (50 μM), but not K_2SO_4, increased the rate of CO_2 uptake substantially (Table I). Low concentrations of LiCl were without effect while high concentrations (30 mM) stimulated CO_2 uptake.

Initiation of CO_2 Transport by Addition of CO_2. Transport of CO_2 can be initiated by illumination (Figs. 2–4) but it can also be initiated by the addition of small volumes of acidified water (40 mM HCl; 1000 times final concentration in cuvette) that have been saturated at 0°C with 5% CO_2 (Fig. 5). As expected, net CO_2 transport in the dark or in the light in the presence of 10 μM DES was substantially inhibited (Fig. 5). Since the response time of measurement, at about 2 s, is large with respect to the time period of maximum uptake (Fig. 5) we have made no attempt to calculate actual rates of CO_2 transport.

Table I. *Effect of Na^+, K^+, and Li^+ on the Rate of CO_2 Uptake by Air-Grown Cells of Synechococcus UTEX 625*

Addition	Rate of CO_2 Uptake[a]
	% of control
Control (buffer)	100
100 μM NaCl	238
50 μM Na_2SO_4	223
50 μM K_2SO_4	116
50 μM LiCl	110
30 mM LiCl	129

[a] The rate of CO_2 uptake was determined prior to (control) and following the addition of the indicated salt. Uptake of CO_2 was initiated by turning on the light. Measurements were made at pH 8.0, 30°C, and 100 μM DIC.

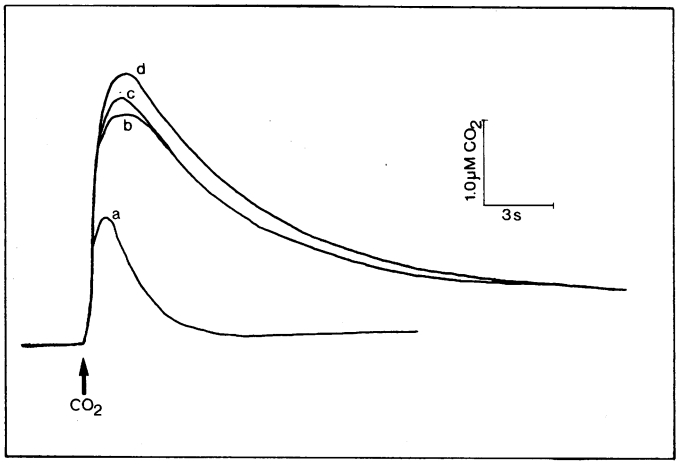

FIG. 5. Measurement of transient CO_2 uptake following a CO_2 pulse. Cells (a, b, c) or buffer alone (d) at pH 8.0 were pulsed with CO_2 by the addition of a small (5 μl) volume of acidified water that had been gassed at 0°C with 5% CO_2. This yielded an initial [CO_2] in the buffer of 4.3 μM. a, Continuously illuminated cells; b, illuminated cells with 10 μM DES; c, darkened cells; d, buffer alone. Due to the about 2 s response time of the mass spectrometer, a full response to the 4.3 μM CO_2 is not observed, even in the absence of cells.

CO_2 Uptake against Concentration Gradient. To determine the extent to which CO_2 uptake can occur against a gradient, we have monitored CO_2 uptake into cells having a high intracellular [CO_2] (Fig. 6). We have previously shown that 20 mM NaCl, at pH 8.0 stimulates the accumulation of large amounts of DIC—and thus CO_2—by the stimulation of HCO_3^- transport (17). In the present experiment (Fig. 6), CO_2 fixation was inhibited by iodoacetamide (26) and $K_2{}^{13}CO_3$ was used, instead of the usual $K_2{}^{12}CO_3$, to avoid ambiguities due to any respiratory release of ^{12}C-DIC into the intracellular and extracellular volumes. Uptake of CO_2 was initiated by illumination of the cells (Fig. 6) and after several minutes 25 mM NaCl was added. The addition of NaCl had no effect upon the steady state uptake of CO_2 (Fig. 6). After sufficient time to allow for accumulation of large amounts of DIC (mainly by HCO_3^- transport under these conditions [17]), CA was added to the cell suspension (Fig. 6). After correction for the enhanced sensitivity of CO_2 measurement in the presence of CA (Fig. 2A), it was determined that only 54.9% of the original ^{13}C remained accessible to the CA. It should be remembered that in the presence of CA, measurement of the CO_2 concentration in the extracellular medium is a reflection of the total DIC concentration because of the very rapid interconversion of CO_2 and HCO_3^-. Assuming that the DIC removed from

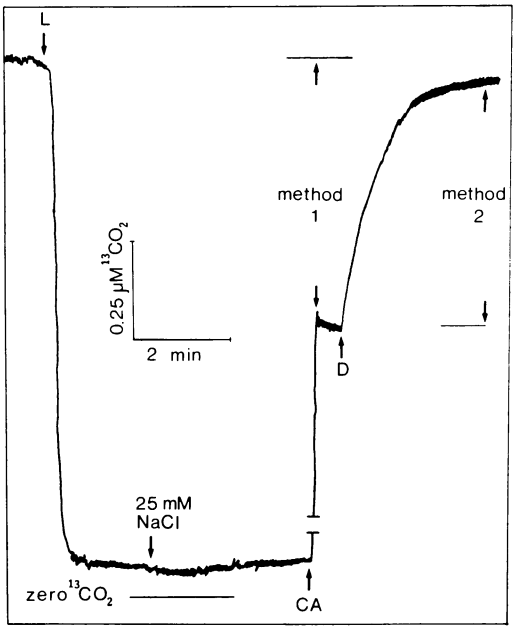

FIG. 6. Transport of CO_2 against a concentration gradient. Cells (15.2 μg Chl/ml) were incubated at pH 8.0 in the presence of 100 μM $K_2{}^{13}CO_3$ and with 3.3 mM iodoacetamide to inhibit CO_2 fixation. At the time indicated 25 mM NaCl was added to initiate HCO_3^- transport (17). CA was added to a final concentration of 25 μg/ml and the lights were turned off (D) at the times indicated. The amount of DIC transported can be estimated by two different methods: Method 1, measurement of amount of DIC removed from the medium by the cells. This is determined by adding CA to the cell suspension in the light. After correction for the enhancement of 17.6% of the mass 44 signal due to CA, the DIC depletion can be estimated as shown. Method 2, upon turning out the lights, the accumulated DIC leaked from the cells. Since CO_2 fixation was inhibited by iodoacetamide a quantitative recovery of the accumulated DIC occurred. $[CO_2]_i$ was calculated as described in "Materials and Methods."

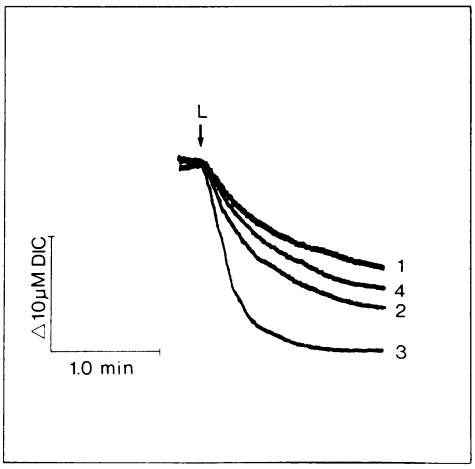

FIG. 7. DIC transport in the presence of CA. Cells (7.9 μg Chl/ml) were incubated in the presence of 50 μM K_2CO_3, 25 μg/ml CA and 3.3 mM iodoacetamide (to inhibit CO_2 fixation). Other additions were made while cells were in the dark and then DIC transport was initiated by turning on the light: 1, no other additions; 2, plus 100 μM NaCl; 3, plus 5 mM NaCl; 4, plus 5 mM NaCl and 20 mM LiCl. The rates of DIC transport were 77, 137, 189, and 87 μmol/mg Chl·h, respectively. The intracellular [DIC] were 20.4, 26.8, 34.9, and 25.5 mM, respectively. Accumulated DIC was quantitatively leaked back into the medium when the light was turned off (data not shown).

the medium was evenly distributed within the intracellular volume, an intracellular DIC concentration of 39.6 mM can be calculated with a corresponding CO_2 concentration of 1.31 mM. The measured extracellular CO_2 concentration just prior to CA addition was only 0.074 μM (Fig. 6) and thus the $[CO_2]_i/[CO_2]_o$ ratio at that time would have been about 17,600 to 1. After the addition of CA the cells were darkened and the accumulated DIC was allowed to leak back into the medium (Fig. 6). Leakage from the cells was slow ($t_{1/2}$ = 80 s) as previously seen with other cyanobacteria (3, 14, 19, 28). Measurement of the amount of ^{13}C-DIC leaking slowly out of the cells in the dark provides another estimate of the intracellular [DIC] that existed during the period of illumination. This measurement gave results very similar to those previously mentioned (42.1 mM total DIC; and a $[CO_2]_i/[CO_2]_o$ ratio of about 18,700 to 1).

CO_2 Uptake in Presence of CA. In the absence of extracellular CA the rate of CO_2 uptake can be limited by the rate at which extracellular HCO_3^- is converted to CO_2 (8, 9, 16, 20). The ability of cells to remove almost all the CO_2 from the medium, while leaving most of the HCO_3^- behind (Fig. 2), is a manifestation of this limitation. The advantages of measuring CO_2 uptake under these conditions were that easily measurable changes in the extracellular CO_2 concentration occurred and a ready distinction between CO_2 and HCO_3^- uptake could be made. However, the addition of CA has been shown to stimulate CO_2 uptake (9, 16, 17) and we have thus carried out measurements under various conditions in the presence of CA (Fig. 7). In the presence of CA, intracellular DIC concentrations were high, as previously reported based upon measurements obtained by the silicone fluid filtration method (17).

Both the rate and extent of DIC accumulation were increased by a low (100 μM) concentration of NaCl (Fig. 7). Uptake of DIC was then further increased by the addition of a high NaCl concentration (5 mM), presumably due to HCO_3^- -transport (17). The addition of 20 mM LiCl reduced the rate and extent of DIC uptake to that expected for CO_2 uptake alone (Fig. 7).

DISCUSSION

Our results show that *Synechococcus* UTEX 625 transports the CO_2 molecule against a large concentration gradient by a process distinct from HCO_3^- transport. This implies the existence of a transport system which recognizes the CO_2 molecule, as distinct from HCO_3^- or CO_3^{2-} and is coupled, directly or indirectly, to the expenditure of metabolic energy. The process we have observed in *Synechococcus* UTEX 625 is the same in these respects to CO_2 uptake by *Anabaena* (1, 2, 9) and the marine *Synechococcus* (2, 3). It is completely distinct from the mere passive movement of CO_2 across biological membranes as a consequence of its lipid solubility (10). The most direct evidence that CO_2 is a substrate for a transport system comes from mass spectrometry (Fig. 2). *Synechococcus* UTEX 625, like the marine *Synechococcus* (2, 3), was able to selectively remove CO_2 from the medium at such a rate that the CO_2 concentration dropped almost to zero while the HCO_3^- concentration remained almost unchanged (Fig. 2). These experiments were facilitated by our finding that HCO_3^- transport in air-grown cells is inhibited in the absence of millimolar concentrations of Na^+ (8, 17). The transport of CO_2 occurred against a large concentration gradient (Fig. 6). Since CO_2 is a weak acid an intracellular accumulation of DIC could occur passively if the cytoplasm were more alkaline than the extracellular medium. This was not the case in our experiments as we purposefully carried our experiments out at pH 8.0, which is higher than the intracellular pH (4, 6). For CO_2 uptake to be driven solely by a pH gradient an intracellular pH of about 12.3 would have been required to account for the in-

organic carbon accumulation ratio observed in the experiment described in Figure 6. Not only is such an intracellular pH not measured when the extracellular pH is 8.0, but the addition of the ionophore monensin, which collapses pH gradients in the presence of Na^+, had no inhibiting effect upon CO_2 uptake (data not shown).

The effects of darkness and the metabolic inhibitors DES and CCCP are consistent with a need for metabolic energy to drive CO_2 uptake (Figs. 2 and 3). Futhermore, the greater effect of DCMU upon O_2 evolution than upon CO_2 uptake (Fig. 4) and the ability of PSI light to support CO_2 uptake suggest an involvement of ATP produced by cyclic or pseudocyclic photophosphorylation. Ogawa and Ogren (25) showed that PSI illumination supported active DIC transport by *Anabaena*. Since their cells were grown on 5% CO_2 rather than air, their cells probably were enriched in CO_2 transport relative to HCO_3^- transport (18). Ogawa *et al.* (26) also found that air-grown cells of *Anacystis nidulans* seemed to require both PSI electron flow itself and the ATP produced in cyclic photophosphorylation. Since their experiments were carried out at pH 7.0 it is also probable that much of the DIC transport was as CO_2 (24).

We have previously reported that low concentrations of Na^+ were required to allow the efficient transport of DIC, apparently as CO_2, by cells grown on high levels (5%) of CO_2 (18). At 500 μM DIC and pH 8.0 a $K_{1/2}$ (Na^+) of 18 μM for photosynthesis was determined (18). The addition of 100 μM NaCl to cells incubated in the absence of added Na^+ served as a simple way to initiate DIC transport (18). Isotopic disequilibrium studies with ^{14}C-DIC indicated that the major form of DIC being transported under these conditions was CO_2 (18). It is now evident that low concentrations of Na^+, but not K^+, stimulate CO_2 transport in air-grown cells as well (Table I). We do not know whether the CO_2 uptake observed in the absence of added Na^+ is truly a Na^+-independent process or whether it is dependent upon the contaminant levels of Na^+ in the incubation solution. A significant stimulation of DIC uptake by 100 μM NaCl was also observed when CO_2 transport was enhanced by the addition of CA (Fig. 7). We previously had thought the CA-stimulated DIC transport was Na^+-dependent (17) but in light of the present results (Table I; Fig. 7) we must now modify this view. In our previous study we examined only the effects of the millimolar Na^+ concentrations known to stimulate HCO_3^- transport (17) as we were not aware then of the effects of micromolar Na^+ on CO_2 transport (18). Our finding that CO_2 transport was not inhibited by high concentrations of Li^+ (17) is confirmed by the present results (Table I). The Na^+-dependent transport of HCO_3^- is inhibited in a competitive fashion by Li^+ (17; GS Espie, AG Miller, DT Canvin, unpublished data). Thus, CO_2 transport, at least at pH 8.0, can be distinguished from HCO_3^- transport by air-grown *Synechococcus* UTEX 625 by its response to micro- rather than millimolar NaCl concentrations and by its resistance to inhibition by LiCl. The site (or sites) of interaction of Na^+ and Li^+ with DIC transport remain unknown.

The observation that the rate of CO_2 uptake remained constant for an extended period of time (Fig. 2) requires comment. There are two possible explanations for this constant rate. First, continued intracellular accumulation of DIC, with no feedback effect on the net rate of CO_2 uptake, could be occurring. Second, net CO_2 uptake could be balanced by an equivalent efflux of HCO_3^-. We designed an experiment to test these two possibilities (Fig. 8). Cells were incubated for 15 min in the light, in the absence of Na^+, with 100 μM $K_2{}^{13}CO_3$. Iodoacetamide was present to inhibit CO_2 fixation. After 15 min, CA was added and then the lights were turned off to allow the accumulated ^{13}C-DIC to leak back into the medium (Fig. 8). From the amount of leakage, the intracellular [^{13}C-DIC] that had existed at the end of the illumination period was calculated at 18.0 mM. This was only 32%

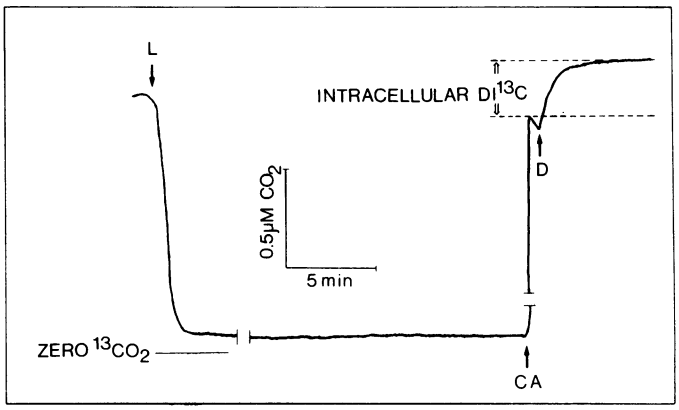

FIG. 8. Evidence for HCO_3^- efflux in the light during CO_2 transport. Cells were incubated with 100 μM $K_2{}^{13}CO_3$ at pH 8.0 in the presence of 3.3 mM iodoacetamide. Only contaminant levels of Na^+ were present. Soon after the addition of 25 μg/ml CA the lights were turned off and accumulated DIC was allowed to leak into the medium. The intracellular [DIC] was calculated from this leakage as being 18.0 mM at the end of the illumination period. The steady state rate of CO_2 transport was calculated from equation 1 where $k_d = 0.87 \times 10^{-3}\ s^{-1}$ and $[HCO_3^-]_i$ half way through the illumination period was estimated as 87.8 μM and $[CO_2]$ was measured as 0.052 μM. The calculated J_{CO_2} was 0.0736 nmol/ml suspension/s (equivalent to a change in $[DIC]_i$ of 62.4 $\mu M \cdot s^{-1}$). The cell density was 15.7 μg Chl/ml. The ΔG for CO_2 leakage is given by $-2.3\ RT \log [CO_2]_i/[CO_2]_o$ and the ΔG for HCO_3^- leakage is given by $-F\Delta\psi + 2.3\ RT \log [HCO_3^-]_i/[CO_2]_o$. A value of -120 mV was used for $\Delta\psi$ (22). The iodoacetamide efficiently inhibited $^{13}CO_2$ fixation, as a total recovery of the added ^{13}C as DIC was obtained (107%) (when the correction for CA enhancement of the mass 45 signal was made).

of the DIC accumulation that should have occurred given the calculated steady state rate of CO_2 transport. Thus, the net uptake of CO_2 must have been offset by a considerable rate of HCO_3^- efflux. There is no reason to doubt that CO_2 efflux also occurs in the light but this experiment (Fig. 8) which monitored only the extracellular [$^{13}CO_2$] gives information only on the net rate of $^{13}CO_2$ uptake. Ogawa and Kaplan (24) have recently come to the same conclusion regarding HCO_3^- efflux during CO_2 uptake by *A. nidulans* R2. They found that HCO_3^- efflux occurred during CO_2 uptake both by wild type cells in which CO_2-fixation was inhibited by iodoacetamide or by mutant cells able to transport CO_2 but unable to effectively fix CO_2 at air concentrations (24). It remains to be seen how much HCO_3^- efflux occurs during CO_2 transport by cells undergoing rapid CO_2 fixation or under conditions (*i.e.* plus Na^+) more favorable to HCO_3^- transport. In the past, thought has been given to the effect of the leakage from the cell of the lipid-soluble CO_2 molecule during DIC transport (2, 3). This remains a valid concern as CO_2 certainly does leak from the cells, at least when the lights are turned off (2; unpublished observations). Badger and Andrews (2) found, however, that the passive permeability to CO_2 of the marine *Synechococcus* really is much lower than would be expected from a consideration of other biological membrane systems. A consideration of the ΔG values for HCO_3^- and CO_2 accumulation (Fig. 8 legend) reveals that the driving force for HCO_3^- leakage is at least as high, at -25.1 kJ/mol, as it is for CO_2 leakage, at -23.2 kJ/mol. Thus, although the accumulation factor for HCO_3^- is usually less than it is for CO_2, this is overcome by the large effect of the negative membrane potential difference—at least -120 mV in the light (22).

The uptake of $^{14}CO_2$ can be measured by the filtration of incubated cells through silicone fluid (1, 2, 29; Fig. 1). Using this technique the effect of inhibitors can be assessed (Fig. 1) but the time resolution prevents its use in detailed kinetic studies.

Consideration of the mass spectrometry results of Figure 5 reveals why the silicone fluid method is inadequate for kinetic studies of CO_2 uptake. The combination of uptake by the cells and the hydration of CO_2 to HCO_3^- in the medium means that by the first sampling time possible with the silicone fluid method (about 14 s) only about 10% of the added CO_2 remains to be taken up. Given this drastic depletion of extracellular CO_2 it is not at all surprising that CO_2 uptake is often essentially complete by the first sampling time (2; Fig. 1). The use of lower cell densities may ameliorate the situation somewhat but we find that with unicellular cyanobacteria, such as *Synechococcus* and *Coccochloris*, that densities below about 6 μg Chl/ml result in a lowered percentage recovery of cells in the terminating solution.

Although *Synechococcus* UTEX 625 can actively transport CO_2 with high affinity, the rate of CO_2 uptake and thus CO_2 fixation, is often limited under experimental conditions by the rate of HCO_3^- dehydration to CO_2 in the extracellular medium (8, 16, 20). This limitation can be overcome, *inter alia*, by increasing the DIC concentration. Thus, for a cell density of 7.8 μg Chl/ml, for example, we have measured a rate of CO_2 uptake of 27 μmol/mg Chl·h at 25 μM DIC (0.4 μM CO_2) while it became 136 μmol/mg Chl·h when the DIC concentration was raised to 350 μM (5.5 μM CO_2) (data not shown). We have previously shown that the inhibition of photosynthesis due to the lack of Na^+ can also be largely overcome by increasing the DIC concentration (8, 17, 22). It is quite possible that the rate of photosynthesis increases as a result of increased CO_2 transport and that HCO_3^- transport remains inhibited, even at high [HCO_3^-], in the absence of Na^+. The limitation on the rate of CO_2 transport can, of course, also be overcome by the addition of CA which increases the rate of CO_2 supply to the transport system (Fig. 7). In this context it is worth noting that there is no evidence that the cyanobacteria examined so far produce their own extracellular CA (2, 3, 8, 9, 13, 20, 21) while many green algae do (7, 23). The function of the extracellular CA of green algae remains a subject of debate (30) and thus its ecological role is unknown. At the low cell densities of cyanobacteria that commonly exist in nature and at mildly alkaline pH it is probable that the dehydration rate of HCO_3^- to CO_2 is not a limiting factor for CO_2 uptake even in the absence of extracellular CA. At pH greater than about 9, where the ability of CA to dehydrate HCO_3^- would be impaired, HCO_3^- uptake provides the majority of DIC for photosynthesis. This capability is likely a major feature in the alkalotolerance of *Synechococcus* UTEX 625, and in conjunction with the active transport of CO_2 provides a diversity of mechanisms for the acquisition of DIC over a large pH range.

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LITERATURE CITED

1. ABE T, M TSUZUKI, S MIYACHI 1987 Transport and fixation of inorganic carbon during photosynthesis of *Anabaena* grown under ordinary air. I. Active species of inorganic carbon utilized for photosynthesis. Plant Cell Physiol 28: 273–281
2. BADGER MR, TJ ANDREWS 1982 Photosynthesis and inorganic carbon usage by the marine cyanobacterium, *Synechococcus* sp. Plant Physiol 70: 517–523
3. BADGER MR, M BASSETT, HN COMINS 1985 A model for HCO_3^- accumulation and photosynthesis in the cyanobacterium *Synechococcus* sp. Theoretical predictions and experimental observations. Plant Physiol 77: 465–471
4. BELKIN S, RJ MELHORN, L PACKER 1987 Proton gradients in intact cyanobacteria. Plant Physiol 84: 25–30
5. BUCH K 1960 Dissoziation der Kohlensaure, Gleichgenichte und Puffersysteme. *In* W Ruhland, ed, Handbuch der Pflanzenphysiologie, Vol I. Springer-Verlag, Berlin, pp 1–11
6. COLEMAN JR, B COLMAN 1981 Inorganic carbon accumulation and photosynthesis in a blue-green alga as a function of external pH. Plant Physiol 67: 917–921
7. COLEMAN JR, AR GROSSMAN 1984 Biosynthesis of carbonic anhydrase in *Chlamydomonas reinhardtii* during adaptation to low CO_2. Proc Natl Acad Sci USA 81: 6049–6053
8. ESPIE GS, DT CANVIN 1987 Evidence for Na^+-independent HCO_3^- uptake by the cyanobacterium *Synechococcus leopoliensis*. Plant Physiol 84: 125–130
9. ESPIE GS, KA GEHL, GW OWTTRIM, B COLMAN 1984 Inorganic carbon utilization by cyanobacteria. *In* C Sybesma, ed, Advances in Photosynthesis Research, Vol III. Martinus Nijhoff/Dr. W Junk, The Netherlands, pp 681–684
10. GUTKNECHT J, MA BISSON, FC TOSTESON 1977 Diffusion of carbon dioxide through lipid bilayer membranes. Effects of carbonic anhydrase, bicarbonate and unstirred layers. J Gen Physiol 69: 779–794
11. HARNED HS, R DAVIS 1943 The ionization constant of carbonic acid in water and solubility of carbon dioxide in water and aqueous salt solutions from 0 to 50°. J Am Chem Soc 65: 2030–2037
12. HOCH G, B KOK 1963 A mass spectrometer inlet system for sampling gases dissolved in liquid phases. Arch Biochem Biophys 101: 160–170
13. KAPLAN A, MR BADGER, JA BERRY 1980 Photosynthesis and the intracellular inorganic carbon pool in the blue-green alga *Anabaena variabilis*: response to external CO_2 concentration. Planta 149: 219–226
14. MARCUS Y, D ZENVIRTH, E HAREL, A KAPLAN 1982 Induction of HCO_3^- transporting capability and high photosynthetic affinity to inorganic carbon by low concentration of CO_2 in *Anabaena variabilis*. Plant Physiol 69: 1008–1012
15. MCENERY MW, PL PEDERSEN 1986 Diethystilbestrol. A novel F_o-directed probe of the mitochondrial proton ATPase. J Biol Chem 261: 1745–1752
16. MILLER AG 1985 Study of inorganic carbon transport: the kinetic reaction approach. *In* WJ Lucas, JA Berry, eds, Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms. American Society of Plant Physiologists, Rockville, MD, pp 17–37
17. MILLER AG, DT CANVIN 1985 Distinction between HCO_3^- and CO_2-dependent photosynthesis in the cyanobaterium *Synechococcus leopoliensis* based on the selective response of HCO_3^- transport to Na^+. FEBS Lett 187: 29–32
18. MILLER, AG, DT CANVIN 1987 Na^+-stimulation of photosynthesis in the cyanobacterium *Synechococcus* UTEX 625 grown on high levels of inorganic carbon. Plant Physiol 84: 118–124
19. MILLER AG, B COLMAN 1980 Active transport and accumulation of bicarbonate by a unicellular cyanobacterium. J Bacteriol 143: 1253–1259
20. MILLER AG, B COLMAN 1980 Evidence for HCO_3^- transport by the blue-green alga (cyanobacterium) *Coccochloris peniocystis*. Plant Physiol 65: 397–402
21. MILLER AG, DH TURPIN, DT CANVIN 1984 Growth and photosynthesis of the cyanobacterium *Synechococcus leopoliensis* in HCO_3^--limited chemostats. Plant Physiol 75: 1064–1070
22. MILLER AG, DH TURPIN, DT CANVIN 1984 Na^+ requirement for growth, photosynthesis and pH regulation in the alkalotolerant cyanobacterium *Synechococcus leopoliensis*. J Bacteriol 159: 100–106
23. MIYACHI S, M TSUZUKI, Y YAGAWA 1985 Carbonic anhydrase in various microalgae. *In* WJ Lucas, JA Berry, eds, Inorganic Carbon Uptake by Aquatic Photosynthesis Organisms. American Society of Plant Physiologists, Rockville, MD, pp 145–154
24. OGAWA T, A KAPLAN 1987 The stoichiometry between CO_2 and H^+ fluxes involved in the transport of inorganic carbon in cyanobacteria. Plant Physiol 83: 888–891
25. OGAWA T, WL OGREN 1985 Action spectra for accumulation of inorganic carbon in the cyanobacterium, *Anabaena variabilis*. Photochem Photobiol 41: 583–587
26. OGAWA T, T OMATA, A MIYANO, Y INOUE 1985 Photosynthetic reactions involved in the CO_2-concentrating mechanism in the cyanobacterium, *Anacystis nidulans*. *In* WJ Lucas, JA Berry, eds, Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms. American Society of Plant Physiologists, Rockville, MD, pp 287–304
27. RIPPKA R, J DERUELLES, JB WATERBURY, M HERDMAN, RY STANIER 1979 Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111: 1–61
28. SHELP BJ, DT CANVIN 1984 Evidence for bicarbonate accumulation by *Anacystis nidulans*. Can J Bot 62: 1398–1403
29. VOLOKITA M, D ZENVIRTH, A KAPLAN, L REINHOLD 1984 Nature of inorganic carbon species actively taken up by the cyanobacterium *Anabaena variabilis*. Plant Physiol 76: 599–602
30. WILLIAMS TG, DH TURPIN 1987 The role of external carbonic anhydrase in inorganic carbon acquisition by *Chlamydomonas reinhardtii* at alkaline pH. Plant Physiol 83: 92–96
31. YOKOTA A, S KITAOKA 1985 Correct pK values for dissociation constant of carbonic acid lower the reported K_m values of ribulose bisphosphate carboxylase by half. Presentation of a nomograph and an equation for determining the pK values. Biochem Biophys Res Commun 131: 1075–1079