Plant Physiol. (1982) 69, 978–982
0032-0889/82/69/0978/05/$00.50/0

Involvement of a Primary Electrogenic Pump in the Mechanism for HCO_3^- Uptake by the Cyanobacterium *Anabaena variabilis*[1]

Received for publication June 5, 1981 and in revised form November 30, 1981

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ABSTRACT

The response of the membrane potential to HCO_3^- supply has been studied in the cyanobacterium *Anabaena variabilis* strain M-3 under various conditions. Changes in potential were followed with the aid of the lipophilic cation tetraphenyl phosphonium bromide.

Addition of HCO_3^- to CO_2-depleted cells resulted in rapid hyperpolarization. The rate and extent of hyperpolarization were greater in low-CO_2-adapted than in high-CO_2-adapted cells. Addition of the electron acceptor *p*-nitrosodimethylaniline which resulted in O_2 evolution in CO_2-depleted cells did not cause hyperpolarization. The hyperpolarization was not attributable to a change in pH or in ionic strength of the medium. Pretreatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea prevented the hyperpolarization. KCN depolarized hyperpolarized cells. Addition of HCO_3^- also brought about immediate K^+ influx which was succeeded after about 2 minutes by K^+ efflux.

Two of the models considered would be capable of explaining these and previous findings: (a) a primary electrogenic pump for transporting HCO_3^- ions; (b) proton-HCO_3^- cotransport, the driving force for which is generated by a proton pump which is sensitive to the HCO_3^- concentration.

Evidence has been accumulating for the operation of an HCO_3^- transporting mechanism in green algae and cyanobacteria, particularly in those adapted to low CO_2 concentrations in their environment (1–3, 6–9, 15, 16, 21, 22, 24). This transporting mechanism results in an increase in CO_2 concentration at the carboxylating site (1, 2, 16, 26) with a consequent increase in apparent photosynthetic affinity for inorganic carbon (1, 3, 11, 16, 23, 26). Decreases in glycolate excretion and photorespiration (3, 10, 13, 17) are also observed during the course of adaptation to low CO_2 conditions. In green algae, the identification of the cell membrane involved in HCO_3^- transport, as well as the extent of accumulation of C_i[2] is complicated by the existence of several subcellular compartments, each with a different volume, a different $\Delta\Psi$ across its boundary membrane, and possibly a different pH. Estimation of the $\Delta\tilde{\mu}$ for HCO_3^- between the external medium and the internal compartment under consideration is consequently very difficult (2, 16). The situation is far less complicated in cyanobacteria since the cell structure is simpler and one may assume that essentially a single internal compartment is involved.

It was previously shown (16) that both high- and low-CO_2-adapted *Anabaena* cells are capable of transporting HCO_3^- and that the difference in apparent photosynthetic affinity for C_i is fully attributable to the different parameters of the HCO_3^- transporting system in the two cases. It was also shown that HCO_3^- transport proceeds against the electrochemical potential gradient for HCO_3^- ions.

The mechanism of HCO_3^- transport, however, is as yet poorly understood. Ferrier (5) and Walker *et al.* (29) have recently suggested that previous findings which were attributed to active HCO_3^- transport in *Chara corallina* (19) could be explained on the basis of CO_2 uptake alone, provided that a proton extrusion pump acidifies a zone adjacent to the cell membrane. Such a mechanism is most unlikely to account for C_i uptake in microalgae as it would not permit accumulation of C_i to the extent reported (16). Further, the operation of the proposed model (5, 29) requires a wide unstirred layer adjacent to the cell membrane which, particularly in unicellular green algae (2), is not likely. In the present paper, we report experiments designed to elucidate the mechanism of HCO_3^- transport in the cyanobacterium *Anabaena variabilis*. Data presented indicate the direct involvement of an electrogenic pump.

MATERIALS AND METHODS

Alga. *Anabaena variabilis* strain M-3 from the collection of Tokyo University was grown at 30°C in a 1-L flask containing Kratz and Myer's medium C (18) supplemented with 10 mM Hepes-NaOH buffer (pH 8.0). Cultures were shaken (50 strokes/min) and aerated with 5% CO_2 (v/v) in air (high CO_2 cells) or air (low CO_2 cells). Continuous illumination was provided by cool-white fluorescent lamps (Tadiran, Israel) at a light intensity of 6 mw cm^{-2} (400–700 nm). Cells were harvested by centrifugation (500*g*; 5 min) and resuspended in 30 mM Hepes-NaOH (pH 8.0), unless otherwise indicated.

K^+ Concentration. This was measured in the medium with a K^+ electrode (model 93-19; Orion Research Inc., Cambridge, MA) inserted into a 50-ml closed vessel containing 22 ml of algal suspension (corresponding to 30 μg Chl/ml) at 30°C (temperature controlled by a water jacket). Incident light intensity on the vessel was 10 mw cm^{-2} (400–700 nm; Halogen-Bellaphot Lamp; Osram, Berlin). The K^+ electrode was calibrated by the addition of known amounts of KCl to the same vessel under the same conditions. The signal from the electrode was amplified and recorded simultaneously with the signal from the O_2 electrode on a dual-channel recorder (model 300; Linear Instruments; Irvine, CA).

O_2 Concentration. This was measured simultaneously with that of K^+ using a Clark-type electrode. The electrode was thoroughly rinsed and checked for a possible leak of KCl by equilibrating the electrodes (O_2 and K^+) in the chamber prior to the addition of cells.

Electric Potential Difference ($\Delta\Psi$). The $\Delta\Psi$ between the cells and the surrounding medium was calculated from the distribution

[1] Supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel.

[2] Abbreviations: C_i, inorganic carbon; TPP^+, lipid-soluble cation tetraphenyl phosphonium; R, ratio of $[^3H]TPP^+$ concentration in cells to the $[^3H]TPP^+$ concentration in medium; PNDA, *p*-nitrosodimethylaniline.

between cells and medium of TPP^+ (phenyl-^3H; obtained from the Nuclear Research Centre, Negev, Israel). *Anabaena* cells were placed in the O_2 electrode chamber (Rank Bros., Bottisham, Cambridge, U. K.) in the light (7 mw cm^{-2}; 400–700 nm) and allowed to utilize the C_i in the medium. TPP^+ was added when CO_2 compensation point (indicated by the cessation of O_2 evolution) was reached. Samples of 150-μl cell suspension were withdrawn periodically, placed in microfuge tubes in the light for an additional 2 min, and then centrifuged (Microfuge B; Beckman Instruments). In experiments involving HCO_3^- supply, $NaHCO_3$ (1 mM) was added either to the microfuge tubes (in short-term experiments) or to the O_2 electrode chamber after TPP^+ had been supplied and samples were periodically withdrawn and treated as described above. The amount of [^{3}H]TPP^+ in 10 μl supernatant and in the pellet was measured in a liquid scintillation counter (Packard Tri-Carb model 3255). The extracellular volume taken down with the cells through the silicone oil layer (60 μl of 4:1 F-50 and RTV-910; Silicone Oil Division, General Electric, Waterford, NY) as well as the total volume (intracellular plus extracellular) were determined as described previously (16, 20).

ATP Concentration. This was determined by the luciferin-luciferase technique. Cells were placed in the O_2 electrode and allowed to utilize the inorganic carbon in the medium. When CO_2 compensation point was reached (indicated by the cessation of O_2 evolution), 1 ml of cell suspension was withdrawn into a syringe containing $HClO_4$ (final concentration, 7%). Cells in the O_2 electrode were then supplemented with 1 mM $NaHCO_3$, and when the rate of O_2 evolution reached steady state, another ml of cell suspension was withdrawn into $HClO_4$. The cell suspension containing $HClO_4$ was neutralized with KOH to pH 7.4 and centrifuged, and the supernatant was analyzed for ATP content. The latter was determined using luminometer 1250-001 and the ATP Kit 1250-120 (LKB Wallack; Stockholm).

RESULTS

Possible Binding of TPP^+ in *Anabaena* Cells. The possibility that TPP^+ binds to sites in *Anabaena* cells, introducing a serious error into estimates of $\Delta\Psi$ across the cell membrane, was investigated by incubating the cells in 0.8 μM [^{3}H]TPP^+ in the presence of different concentrations of unlabeled TPP^+. Competition of unlabeled and labeled TPP^+ for binding sites would result in a fall in R with increasing concentrations of unlabeled TPP^+. Data presented in Figure 1 clearly show such a drop in R with increasing concentration of unlabeled TPP^+ up to 5 μM. Above this concentration, however, R is not further affected. This may indicate saturation of binding sites for TPP^+. Further, it indicates that TPP^+ concentrations in the range of 5 to 20 μM do not influence the $\Delta\Psi$ across the cell membrane. Experiments were accordingly carried out at a TPP^+ concentration of 8 μM unlabeled plus 0.8 μM [^{3}H]TPP^+ (final specific activity, 251 mCi/mmol). Neither the rate of photosynthesis nor HCO_3^- uptake were affected by this concentration of TPP^+.

Hyperpolarization in Response to HCO_3^- Supply. *Anabaena* cells suspended in 30 mM Hepes-NaOH buffer (pH 8.0) were placed in the O_2 electrode chamber and allowed to utilize the C_i present until the rate of O_2 evolution reached zero (CO_2 compensation point). [^{3}H]TPP^+ was then added after which aliquots of cell suspension were removed periodically and transferred to microfuge tubes previously flushed with N_2. Incubation with [^{3}H]TPP^+ was terminated by centrifugation. Figure 2 indicates that the distribution of TPP^+ between cells and medium reached equilibrium in less than 5 min exhibiting a membrane potential of 74 mv (negative inside). The addition of $NaHCO_3$ (1 mM in 10 mM Hepes-NaOH, pH 8.0) to CO_2-depleted cells (Fig. 2, arrow) resulted in immediate hyperpolarization, as indicated by enhanced TPP^+ uptake. This effect was observed both for high- and low-CO_2-adapted cells. Both the rate and extent of TPP^+ uptake were higher in the latter case. Maximum hyperpolarization was reached after 90 to 120 s in the presence of HCO_3^- which is also the time taken to reach a steady-state internal C_i concentration (16). The addition of NaCl in place of $NaHCO_3$ did not result in uptake of TPP^+ (Fig. 2, open circles). If DCMU (5×10^{-6} M) was added to the cells 2 min before the addition of HCO_3^-, the HCO_3^--induced

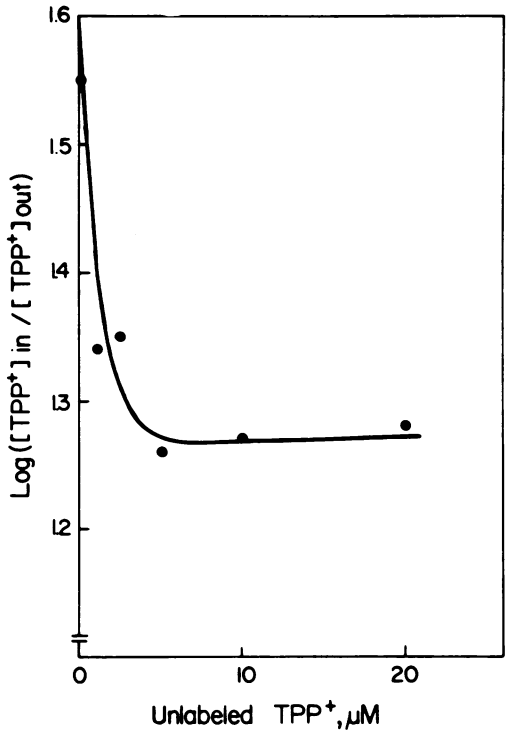

FIG. 1. The distribution of [^{3}H]TPP^+ between *Anabaena* cells and medium as affected by concentration of unlabeled TPP^+. Cells were exposed to TPP^+ for 10 min in the absence of CO_2 at 30°C, light intensity 6 mw cm^{-2} (400–700 nm).

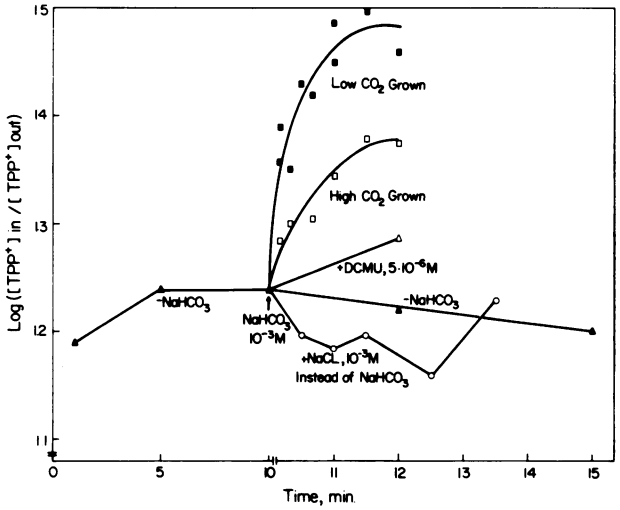

FIG. 2. Distribution of TPP^+ between *Anabaena* cells and medium as affected by various treatments. TPP^+ (8 μM unlabeled + 0.8 μM [^{3}H]TPP^+) added at time zero to cells depleted of CO_2 in the O_2 electrode chamber. $NaHCO_3$ (1 mM) was supplied after 10 min to low- (■) and high- (□) CO_2-grown cells. (△), DCMU (5×10^{-6} M) added to low-CO_2-grown cells 20 s before the addition of HCO_3^-; (○), NaCl (1 mM) added to low-CO_2-grown cells in place of $NaHCO_3$; (▲), no addition of HCO_3^-. Note the break and change in the time scale. Other conditions as in Figure 1.

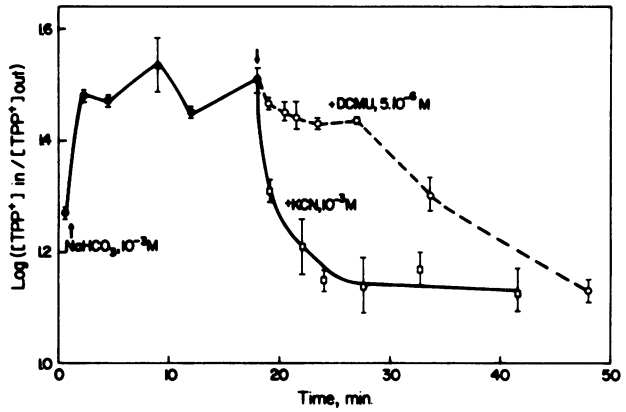

FIG. 3. Distribution of TPP^+ between *Anabaena* cells and medium as affected by HCO_3^- supply, DCMU, and KCN. CO_2-depleted cells were incubated in the presence of [^{3}H]TPP^+ for 5 min before the addition of $NaHCO_3$ (1 mM; ●). KCN (1 mM; □) and DCMU (5×10^{-6} M; ○) added where indicated.

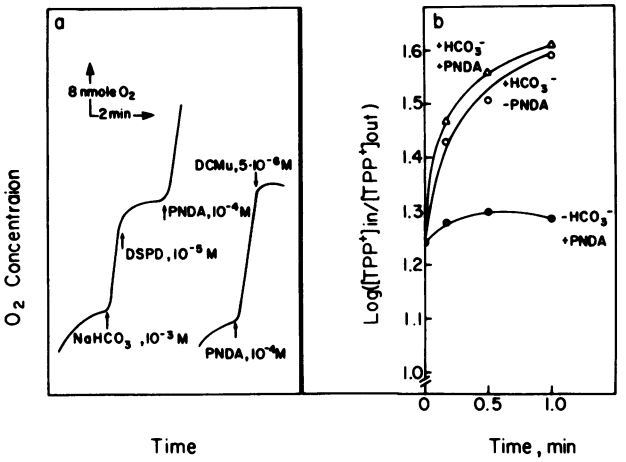

FIG. 4. Response of O_2 evolution (A) and distribution of TPP^+ (B) to PNDA. A, Three ml of *Anabaena* cells, corresponding to 10 μg Chl/ml, in the O_2 electrode. Light intensity was 10 mw cm^{-2} (400–700 nm), 30°C. B, PNDA (10^{-4} M) and $NaHCO_3$ (10^{-3} M) added (where indicated) at time zero into the microfuge tube containing the cell suspension.

TPP^+ uptake was severely reduced (Fig. 2, open triangle). The same treatment inhibited HCO_3^- uptake in *Anabaena* by 90% (not shown).

In the experiment presented in Figure 3, the HCO_3^--induced hyperpolarization was maintained, with certain fluctuations in potential, for at least 18 min. Addition of 1 mM KCN after 18 min resulted in a rapid fall in potential. On the other hand, addition of DCMU under these conditions (as opposed to those of Fig. 2, where DCMU was added before the HCO_3^-) produced a much slower decay in potential.

A possibility to be considered was that the HCO_3^--induced hyperpolarization resulted from the stimulation of photosynthetic electron transport consequent on HCO_3^- supply to CO_2-depleted cells. To test this possibility, the electron acceptor PNDA (4) was supplied to intact *Anabaena* cells which had been depleted of CO_2 (as indicated by the cessation of O_2 evolution). PNDA was chosen because the activity of other PSI electron acceptors such as methylviologen results in formation of peroxides which severely inhibits HCO_3^- uptake in cyanobacteria (15). Figure 4A shows that the rate of PNDA-dependent O_2 evolution was similar to the rate of CO_2-dependent O_2 evolution at saturating CO_2 concentration. PNDA-dependent O_2 evolution is inhibited by DCMU (and dibromomethyl-isopropyl-*p*-benzoquinone, not shown) but is not affected by disalicylidene propanediamine which completely inhibited CO_2-dependent O_2 evolution. However, no hyperpolarization was observed, *i.e.* the distribution of TPP^+ between cells and medium was scarcely affected by PNDA (Fig. 4B). When HCO_3^- is supplied, hyperpolarization immediately occurred with no marked effect of PNDA (Fig. 4B). At saturating light intensity (10 mw cm^{-2}; 400–700 nm) the rates of HCO_3^- uptake and CO_2 fixation were essentially not affected by the PNDA concentration used here (not shown). This indicates that the presence of PNDA does not alter the formation of ATP which is coupled to electron transport. ATP pool itself was hardly affected by the presence or absence of HCO_3^- (178 and 190 nmol ATP/mg Chl, respectively).

K^+ Flux in Response to HCO_3^- Supply. When HCO_3^- was added to cells depleted of CO_2 as indicated by the cessation of O_2 evolution (14), a marked influx of K^+ was immediately observed (Fig. 5). K^+ was taken up for about 2 min after which it was released. The rate of O_2 evolution remained constant during that time. In a parallel experiment carried out in an unbuffered medium, alkalization of the medium accompanying the utilization of HCO_3^- in photosynthesis was measured instead of O_2 evolution (not shown). After a lag in alkalization which resembled that previously reported (14), alkalization of the medium proceeded at a constant rate similar to that observed for O_2 evolution (Fig. 5).

The uptake of K^+ could conceivably be interpreted as indicating a symport of K^+ and HCO_3^-. This possibility was checked in an experiment where the dependence of photosynthetic rate on external K^+ concentration was evaluated. The rationale for this experiment was as follows: photosynthetic rate at low C_i concentration is limited by the HCO_3^- transporting capability of the cells (16). If HCO_3^- transport is compulsorily linked with K^+ transport, then the rate of photosynthesis at low external [HCO_3^-] should be affected by external [K^+]. Figure 6, however, clearly shows that the dependence of photosynthesis on C_i was not influenced by external [KCl]. Though the maximum rate of photosynthesis was slightly decreased by KCl concentrations above 20 mM (not shown), KCl concentrations up to 20 mM had no effect on the apparent photosynthetic affinity to C_i. It should be noted that, in these experiments, cells were suspended in a mixture of Hepes and bis-Tris propane buffer, to avoid inclusion of the NaOH present in the standard buffer, since Na^+ might conceivably be capable of

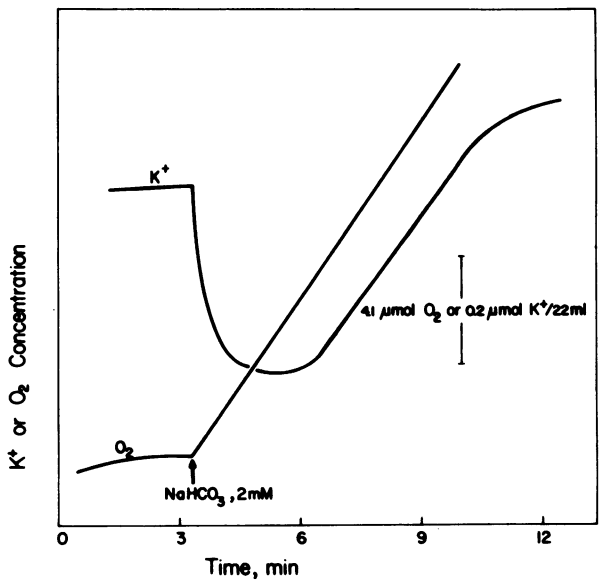

FIG. 5. Net fluxes of O_2 and K^+ in response to the addition of HCO_3^- to CO_2-depleted *Anabaena* cells. Cell suspension (22 ml; 30 μg Chl/ml), 30°C, light intensity 10 mw cm^{-2} (400–700 nm).

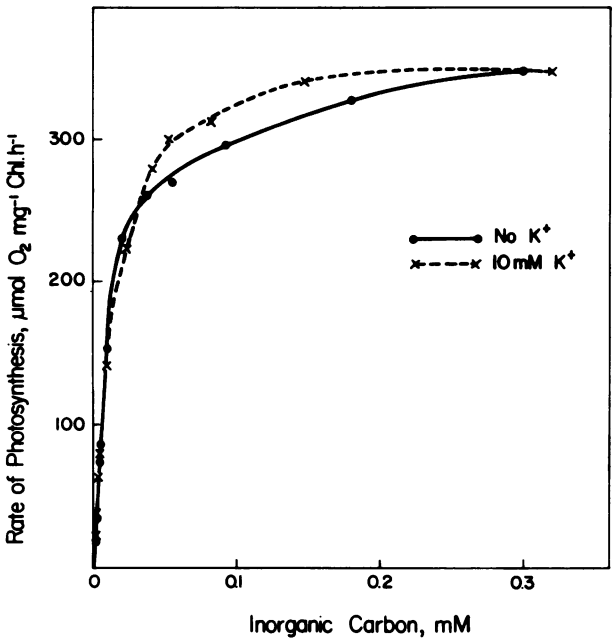

FIG. 6. Rate of photosynthesis as a function of external C_i concentration in the presence and absence of 10 mM KCl.

substituting for K^+ in the putative K^+-HCO_3^- symport. The photosynthetic response of *Anabaena* to the $[C_i]$ was similar to that observed in Hepes-NaOH buffer.

The lack of dependence of KCl concentration also indicates that hyperpolarization and transport of Cl^- are not causally related.

DISCUSSION

Data presented here clearly show that the addition of HCO_3^- to CO_2-depleted cells results in an immediate uptake of TPP^+. This influx of TPP^+ most probably reflects hyperpolarization of the cell membrane potential. The other alternative to be considered is a change in the degree of binding of TPP^+ to some cell component in response to $NaHCO_3$ supply. The second alternative seems to be the less likely, since, first, the binding sites, if any, were apparently saturated at the TPP^+ concentration employed (Fig. 1); and second, ionic strength *per se* is not the basis of the HCO_3^- effect, since NaCl failed to increase uptake of TPP^+ (Fig. 2). Our conclusion that TPP^+ uptake in response to HCO_3^- addition in all likelihood reflects hyperpolarization is consonant with the wide use of lipid-soluble cations such as TPP^+ for determination of $\Delta\Psi$ in algae and cyanobacteria (25, 27).

It has been reported (6, 24) that the presence of HCO_3^- inhibits Cl^- uptake, but not vice versa. The HCO_3^--induced hyperpolarization reported here may well provide the explanation for the depression of Cl^- uptake without invoking competition for carrier sites. The lack of the reverse effect may also be readily understood on this basis.

The initial strong K^+ influx upon addition of HCO_3^- to CO_2-depleted cells (Fig. 5) may also reflect the hyperpolarization of the membrane. (Note that the time which elapsed before maximum hyperpolarization was reached was similar to that required to achieve the steady-state internal C_i pool size [16] and maximal internal K^+ concentration [Fig. 5].) The subsequent K^+ efflux observed might be due to an outwards K^+-OH symport (24), or a K^+ in/OH^- out antiport.

HCO_3^--induced hyperpolarization could theoretically result from one or more of the following causes:

(a) Alteration of the external pH due to the addition of $NaHCO_3$. Spanswick (28) has demonstrated that the reported hyperpolarization of the cell membrane of *Chara* following the addition of HCO_3^- (12) may have been due to alteration of the external pH. The pH of the medium strongly affects the distribution of TPP^+ between *Anabaena* cells and medium (26; A. Kaplan, manuscript in preparation). In the experiments reported here, however, $NaHCO_3$ was prepared in Hepes-NaOH buffer (pH 8.0) and the pH of the medium was constant throughout. This possible cause may therefore be discounted.

(b) Enhancement of photosynthesis when HCO_3^- is supplied to CO_2-depleted cells. Enhanced activity of redox pumps is unlikely to have been the cause of the observed hyperpolarization since, in the presence of PNDA, electron transport operated at a rate similar to that obtained in the presence of HCO_3^- (Fig. 4A). Yet, hyperpolarization was not observed unless HCO_3^- was present (Fig. 4B).

The possibility should be considered that the hyperpolarization in response to HCO_3^- supply is related to enhancement of electrogenic pumps as a result of greater availability of ATP at high photosynthetic rate. While this possibility cannot be excluded, it seems unlikely in view of the facts that (a) the pool size of ATP was hardly affected by the addition of HCO_3^- to CO_2-depleted cells; and (b) though the extent of coupling between PNDA-dependent electron transport and phosphorylation was not determined, the lack of response of CO_2 fixation and HCO_3^- uptake, at saturating light intensity, to the presence of PNDA give no indication to PNDA acting as an uncoupler. Thus, it seems likely that, should ATP availability limit electrogenic pumps and thus hyperpolarization, this requirement should be satisfied when PNDA is supplied to CO_2-depleted cells. A strong indication that the hyperpolarization did not mainly reflect the rate of photosynthesis is provided by Figure 2. At a saturating HCO_3^- concentration (the conditions of the experiment shown in Fig. 2), high- and low-CO_2-grown cells photosynthesized at the same rate (16). The rate and extent of hyperpolarization, however, was greater in the case of low-CO_2-grown cells (Fig. 2). Low-CO_2-grown cells also take up HCO_3^- much faster than do high-CO_2-grown cells (16). The HCO_3^--induced hyperpolarization would thus seem to be associated with HCO_3^- uptake.

Beardall and Raven (2) have recently proposed that a primary electrogenic HCO_3^- pump may be involved in the 'CO_2-concentrating mechanism' in *Chlorella*. A crucial issue is to decide between this possibility and alternative models.

Any model proposed to explain HCO_3^- uptake and accumulation must take account of the following findings: (a) uptake of HCO_3^- against its electrochemical potential gradient (14); (b) hyperpolarization of the membrane potential in response to HCO_3^- supply (Figs. 2, 3, and 4B); (c) the K^+ flux following HCO_3^- supply (Fig. 5); (d) continuous alkalization of the medium during photosynthesis (14) at a rate similar to that of O_2 evolution; (e) lag in alkalization immediately following HCO_3^- supply (14). (f) response to various inhibitors (Figs. 2 and 3), and the marked depression of HCO_3^- uptake in the dark (A. Kaplan, manuscript in preparation).

A possibility that should be briefly considered is that of a HCO_3^-/OH^- antiport system (8, 21). This possibility must be rejected, however, because first, it would not account for hyperpolarization; second, it does not explain the lag observed in alkalization of the medium; and third, it would require fixed HCO_3^-/OH^- stoichiometry, and the latter is in fact pH dependent (14).

A model which readily suggests itself in the light of Mitchell theory is that a primary, ATP-fueled proton extrusion pump provides a proton motive force directed inwards, and that HCO_3^- ions and protons are cotransported inwards, driven by the proton motive force. This model would account for 'uphill' HCO_3^- uptake and the observed responses to inhibitors. Beardall and Raven (2)

regarded proton-HCO_3^- cotransport as unlikely, because on energetic grounds more than one proton per HCO_3^- is required, and cotransport would thus lead to depolarization and not hyperpolarization. We suggest that hyperpolarization might, however, result, provided that the rate of proton pumping is increased in the presence of HCO_3^-. Alkalization of the medium observed on addition of HCO_3^- to CO_2-depleted cells might be due to OH^- uniport outwards, provided the latter is downhill along its electrochemical potential gradient. (A downhill gradient is likely since, in *Anabaena* held at an external pH of 8.0, $\Delta\Psi$ is approximately 90 mv, negative inside [Figs. 2, 3, and 4B] and internal pH is approximately 7.8 [26; A. Kaplan, unpublished data].) The lag in alkalization might reflect a slight delay before OH^- produced from HCO_3^- reaches the exterior.

Another possibility worth consideration is that alkalization is due to outwards K^+-OH^- symport (see above). The lag in alkalization might be due to a prompter response of the putative proton extrusion pump to HCO_3^- addition as compared with the K^+-OH^- symporter. However, the lack of response of photosynthesis to K^+ concentration (Fig. 6) indicates that the steady-state OH^- efflux (resulting from HCO_3^- utilization in photosynthesis) cannot be compulsorily linked to K^+ efflux.

A second model for HCO_3^- uptake would envisage HCO_3^- transport itself as mediated by a primary electrogenic pump, and not as secondary to proton transport. Alkalization of the medium would again be the result of exit of OH^- ions via a uniport system.

Both of the above models (H^+-HCO_3^- symport secondary to an H^+ extrusion pump, and a primary HCO_3^- pump) are capable of explaining the experimental findings. The data at present are not sufficient to enable decisive choice between them. However, the requirement of the first model for a raised steady-state rate of proton extrusion is difficult to accommodate with concomitant alkalization of the external medium. The second model would thus seem to be the more likely. Both models seem to imply the existence of a HCO_3^- stimulated membrane ATPase; and experiments are now in progress in our laboratory to explore this possibility.

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