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Evidence Against H^+-HCO_3^- Symport as the Mechanism for HCO_3^- Transport in the Cyanobacterium *Anabaena variabilis*

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Summary. The rate of inorganic carbon uptake and its steady-state accumulation ratio (intracellular/extracellular concentration) was determined in the cyanobacterium *Anabaena variabilis* as a function of extracellular pH. The free energy of protons ($\Delta\tilde{\mu}_{H^+}$) across the plasmalemma was calculated from determinations of membrane potential, and intracellular pH, as a function of the extracellular pH. While inward proton motive force decreased with increasing extracellular pH from 6.5 to 9.5, rate of HCO_3^- influx and its accumulation ratio increased. The latter is several times larger than would be expected should HCO_3^- influx be driven by $\Delta\tilde{\mu}_{H^+}$. It is concluded that HCO_3^- transport in cyanobacteria is not driven by the proton motive force.

Key Words *Anabaena* · bicarbonate transport · cyanobacteria · photosynthesis · proton motive force

Introduction

The mechanism of active HCO_3^- transport in cyanobacteria and green algae [*see* 11, 12 for references] is not yet understood. Two models have been proposed to explain the active accumulation of inorganic carbon (C_i) and the HCO_3^--induced hyperpolarization of the cell membrane [11]: (a) A primary electrogenic pump for HCO_3^- [2, 11] and (b) H^+-HCO_3^- symport or OH^-/HCO_3^- antiport [5, 14] secondary to an H^+ extrusion pump [11]. In the latter model HCO_3^- is envisaged as driven by the difference in free energy of H^+ across the membrane ($\Delta\tilde{\mu}_{H^+}$, which is the sum of ΔpH and $\Delta\psi$). Hyperpolarization on addition of HCO_3^- will be observed if the H^+ extrusion pump is stimulated by the presence of HCO_3^-. An analysis of the extent of accumulation of inorganic carbon within the cells as a function of $\Delta\tilde{\mu}_{H^+}$ should enable critical assessment of the validity of this model.

$\Delta\psi$ and ΔpH may be modified by various treatments, such as raising the concentrations of K^+ [18] or a lipophilic cation [8] in the medium. The approach taken here was to study HCO_3^- transport in response to the pH in the medium. The intracellular pH of *Anabaena variabilis* [17] and *Coccochloris peniocystis* [3] is only slightly affected by the external pH over the range of 6.5–8 and 7.0–10.0, respectively. Consequently, ΔpH is strongly affected by the external pH. Hyperpolarization of the cell membrane has been observed in *A. variabilis* in response to elevated pH [17]. It is too small, however, to result in maintenance of $\Delta\tilde{\mu}_{H^+}$ at a constant value over a large range of external pH. Therefore $\Delta\tilde{\mu}_{H^+}$ decreases as external pH is elevated from 6.0 to 8.0 [17]. Similar results were obtained in *E. coli* [*see* 15]. Since $\Delta\tilde{\mu}_{H^+}$ is considered to be the driving force for HCO_3^- transport according to the H^+-HCO_3^- symport model, raising the external pH should lower the HCO_3^- transport capability, and hence the accumulation ratio ($[C_i]_{in}/[C_i]_{out}$), unless the parameters of $\Delta\tilde{\mu}_{H^+}$ have a strong effect on the kinetics of transport [7]. In the study presented here we determined the effect of external pH and $\Delta\tilde{\mu}_{H^+}$ on the one hand and on the transport of HCO_3^- and its accumulation ratio on the other.

Materials and Methods

Cells of *Anabaena variabilis* strain M-3 from the collection of the Tokyo University were grown aerated with air as described elsewhere [11]. Rate of oxygen exchange and accumulation of inorganic (acid labile) carbon were determined by means of a O_2 electrode and the filtering centrifugation technique, respectively, as described earlier [10]. The pH of the medium was altered by suspending the cells in mixtures of N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), N-2-hydroxyethylpiperazine propane sulfonic acid (EPPS) and 1,3-bis(tris hydroxymethyl)methylamino propane (BTP) (25 mM each). The required pH was obtained by titration with NaOH. Intracellular pH was determined essentially as described by Coleman and Colman [3] using the distribution between cells and medium of 5,5-dimethyloxazolidine-2,4-Dione (DMO), at pH's 6.5–8.0 and methylamine at pH's 8.0–9.5. Uptake of the charged species of methylamine in addition to the uncharged would be expected to result in un-

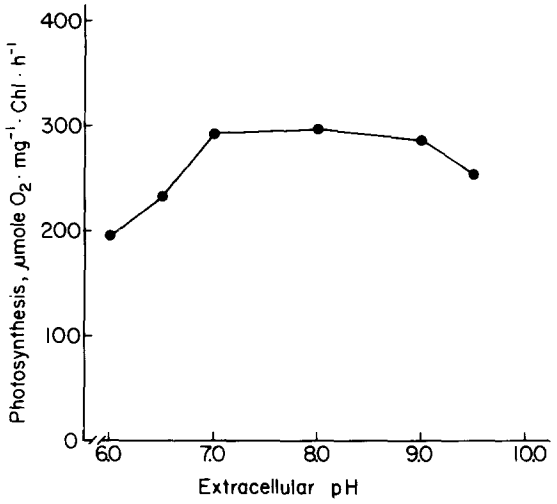

Fig. 1. Photosynthetic O_2 evolution in *A. variabilis* as a function of extracellular pH. Two ml of cell suspension corresponding to 7.0 μg Chl/ml in the O_2 electrode. 30°C, 6.5 mW · cm^{-2} (400–700 nm) saturating extracellular inorganic carbon concentration (1 mM). Data presented are average of three replicates. Variability was smaller than ±10% of the average

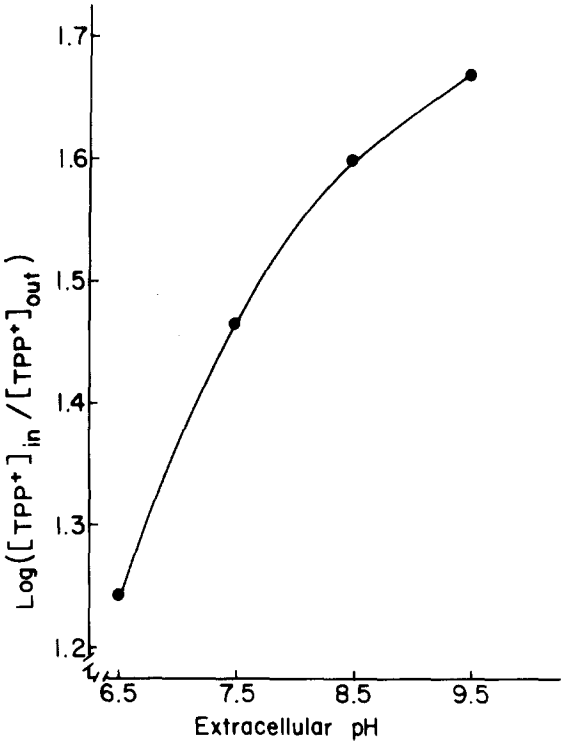

Fig. 2. Distribution of tetraphenylphosphonium (TPP^+) between cells and medium as a function of extracellular pH. Extracellular TPP^+ concentration 15 μM, 30°C, 0.5 mM $NaHCO_3$, 6.0 mW · cm^{-2} (400–700 nm). Data presented are the average of six replicates. Variability was ±5–10% of the average

derestimation of the cytoplasmic pH. This would be most pronounced at pH 8.0 where the methylammonium over methylamine concentration ratio is the largest (for the pH range in which methylamine was used). The time course of methylamine uptake (not shown) indicated two main phases. The first phase of rapid uptake was completed after about 5–10 min and was followed by prolonged uptake at reduced rate. We attribute the first phase to uptake of methylamine, and the second phase which was most pronounced at pH 8.0 to uptake of methylammonium. Extrapolation of the second phase to zero time gave an estimate of the uptake of the uncharged species only. When extracellular pH was 8.0 the estimated cytoplasmic pH was 7.8 and 7.7 with DMO or methylamine, respectively. The membrane potential was determined from the distribution of tetraphenylphosphonium (TPP^+) as described earlier [11].

Results and Discussion

The rate of O_2 evolution at saturating levels of inorganic carbon (C_i) was scarcely affected by the external pH over the range of 7.0 to 9.0 (Fig. 1). This finding is in agreement with our previous report [9] and the results of Coleman and Colman [3] in *Coccochloris*. It may serve as another indication that there is little or no change in internal pH because at saturating C_i level the activity of ribulose 1-5-bisphosphate (RuP_2) carboxylase (which is strongly pH dependent [3]) limits the rate of photosynthesis (M. Volokita, *in preparation*).

The $\Delta\psi$ across the cell membrane as calculated from the distribution of tetraphenylphosphonium ion (TPP^+) is shown in Fig. 2 as a function of the external pH. Hyperpolarization of cell membrane is observed as the external pH is raised from 7.0 to 9.0. The data presented in Fig. 2 are those obtained in the presence of 0.5 mM C_i. The extent of the C_i induced hyperpolarization [11] was scarcely affected by external pH over the range used here (not shown).

The initial rate of C_i uptake, following HCO_3^- supply, as a function of external concentration, at different pH values is shown in Fig. 3. These data were calculated from experiments in which the time course of accumulation of C_i within the cells was followed at various pH's (not shown). Analysis of the data in Fig. 3 indicates that the HCO_3^- transporting system does not conform to Michaelis-Menten kinetics. This is especially pronounced at pH 8.5 and 9.5. The parameters of $\Delta\tilde{\mu}_{H^+}$ (ΔpH and $\Delta\psi$) are energetically equivalent. They may, however, influence kinetic parameters differently [7]. The effect of extracellular pH, ΔpH and $\Delta\psi$ on kinetic parameters of HCO_3^- transport in *Anabaena* cells will be discussed separately.

The intracellular steady-state pool of C_i depends on the rates of its influx and of its dissipation.

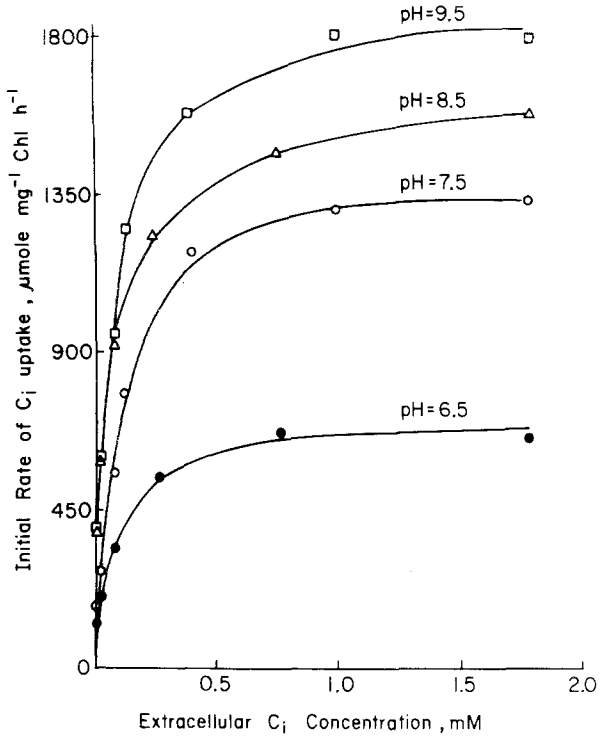

Fig. 3. Initial rate of inorganic carbon (C_i) uptake as a function of its external concentration at different pH values. Rate determined from the slope of curve relating accumulation of C_i 5 and 10 sec after the addition of $NaH^{14}CO_3$ at different concentrations. Three replicates, variability less than ±10% of the presented average

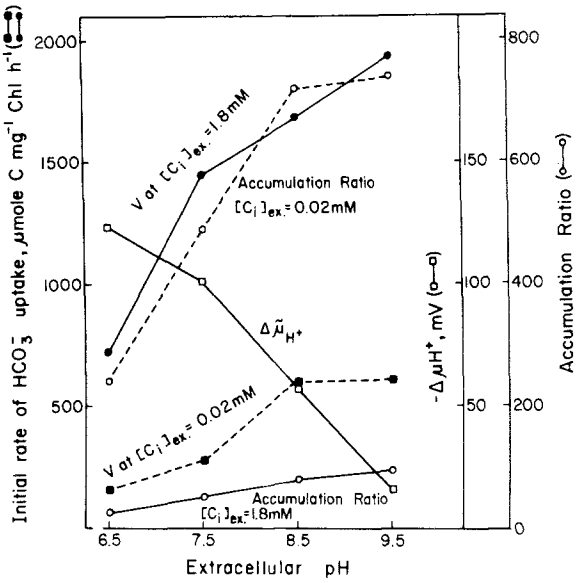

Fig. 4. Initial rate of HCO_3^- uptake (V) accumulation ratio and $\Delta\tilde{\mu}_{H^+}$ as a function of extracellular pH. V and the accumulation ratios are given at limiting (0.02 mM) and saturating (1.8 mM) extracellular inorganic carbon concentration ($[C_i]_{ex}$). Data for accumulation ratio are taken from the steady-state intracellular C_i concentration determined in time course experiments following the supply of C_i to CO_2-depleted cells. $\Delta\tilde{\mu}_{H^+}$ calculated from membrane potential (Fig. 2) and measurement of intracellular pH as a function of extracellular (not shown but very similar to that reported in *Coccochloris* [3])

The latter is due to efflux of C_i and its photosynthetic utilization. The rate of efflux of C_i was measured at pH 8.0 only [13]. Data obtained indicated that the rate of efflux of C_i is rather small compared to the initial rate of influx (420 and 1400 μmole C_i · mg^{-1} Chl · h^{-1}, respectively). The maximal rate of photosynthetic O_2 evoluation was not affected by pH over the range of 7.0–9.0 (Fig. 1). Thus the photosynthetic dissipation of the intracellular C_i pool must be constant over this pH range (~300 μmol C_i · mg^{-1} Chl · h^{-1}). It is concluded that the rate of dissipation of intracellular C_i (via efflux and photosynthesis) is about half the initial rate of C_i influx. This must indicate that the steady-state influx rate is slower than the initial rate, perhaps due to some inhibition on influx exerted by the intracellular C_i pool. The effect of the intracellular C_i pool on unidirectional C_i fluxes is now being studied. The C_i accumulation ratio was determined from experiments in which the time course of accumulation of the intracellular C_i pool were followed as affected by external pH between 6.5 and 9.5 (not shown, but *see* [10] for time course at pH 8.0). It should be emphasized that maximum accumulation was reached after 90–120 sec in the case of extracellular C_i concentration of 1.8 mM and within 30–60 sec in the case of 0.02 mM. In the latter case the intracellular pool decreased after 90 sec due to utilization of extracellular inorganic carbon [10].

As pointed out in the introduction, should the H^+-HCO_3^- symport model be correct, the driving force for C_i uptake would be $\Delta\tilde{\mu}_{H^+} + \Delta\tilde{\mu}_{C_i}$. Maximum accumulation ratio of C_i and the resulting $\Delta\tilde{\mu}_{C_i}$ should not exceed $\Delta\tilde{\mu}_{H^+}$. In Fig. 4 the rates of C_i uptake, its accumulation ratio, and the $\Delta\tilde{\mu}_{H^+}$ (calculated from Fig. 2 and intracellular pH measurements) are plotted against the extracellular pH. The data clearly indicate that as the hypothetical driving force for inward H^+ movement decreases the maximum rate of C_i uptake and the accumulation ratio are increasing. Further, at pH 9.5, for example, $\Delta\tilde{\mu}_{H^+}$ is only about −15 mV, where $\Delta\tilde{\mu}_{C_i}$ corresponds to about 260 mV at extracellular C_i of 0.02 mM and 210 mV at 1.8 mM extracellular C_i. This is just the opposite of what would be expected should the H^+-HCO_3^- symport model be correct. HCO_3^- influx driven by $\Delta\tilde{\mu}_{H^+}$, would require a stoichiometry of about 17 H^+ per HCO_3^-. This would be very

difficult to reconcile with the observed hyperpolarization on addition of HCO_3^- [11]. It is therefore concluded that HCO_3^- transport and accumulation in cyanobacteria is not driven by the proton motive force. Therefore the other alternative of primary electrogenic pump for HCO_3^- seems preferable and is now being examined further.

Alkalization of the cytoplasm has been suggested as the cause for inhibition of photosynthesis in *Anabaena* at pH's higher than 9.5 [9]. This may be explained by the fall of $\Delta\tilde{\mu}_{H^+}$ (Fig. 4) as the latter may serve as the driving force for efflux of OH^- intracellularly released from HCO_3^- (during utilization of CO_2 in photosynthesis). Furthermore, uptake of different solutes [16] and activity of enzymes such as nitrogenase [6] is thought to depend on $\Delta\tilde{\mu}_{H^+}$ and its parameters. $\Delta\tilde{\mu}_{H^+}$ may therefore determine the upper pH limit for growth of cyanobacteria.

It is interesting to note that the accumulation ratio at a given extracellular C_i concentration does not correlate well with the initial rate of C_i uptake (Fig. 4). Theoretically the accumulation ratio should not be affected by the extracellular concentration. It is often observed, however, as is also the case here (Fig. 4), that the accumulation ratio falls with increasing extracellular substrate concentration. Eddy [4] has recently put forward several possible explanations of such findings, and we are now attempting to analyze the case for inorganic carbon accumulation in cyanobacteria.

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