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Inorganic Carbon Accumulation and Photosynthesis in a Blue-green Alga as a Function of External pH[1]

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ABSTRACT

The blue-green alga *Coccochloris peniocystis* photosynthesizes optimally over the pH range of 7.0 to 10.0, but the O_2-evolution rate is inhibited below pH 7.0 and ceases below pH 5.25. Measurement of the inorganic carbon pool in this alga in the light, using the silicone-fluid filtration technique demonstrated that the rate of accumulation of dissolved inorganic carbon remained relatively constant over a wide pH range. At external dissolved inorganic carbon concentrations of 0.56 to 0.89 millimolar the internal concentration after 30 seconds illumination was greater than 3.5 millimolar over the entire pH range. Intracellular pH measured in the light using [^{14}C]5,5-dimethyloxazolidine-2,4-dione and [^{14}C]methylamine dropped from pH 7.6 at an external pH of 7.0 to pH 6.6 at an external pH of 5.25. Above an external pH of 7.0 the intracellular pH rose gradually to pH 7.9 at an external pH 10.0. Ribulose-1,5-bisphosphate carboxylase activity of cell-free algal extracts exhibited optimal activity at pH 7.5 to 7.8 but was inactive below pH 6.5. It is suggested that the inability of *Coccochloris* to maintain its intracellular pH when in an acidic environment restricts its photosynthetic capacity by a direct pH effect on the principal CO_2 fixing enzyme.

Blue-green algae generally have been found in alkaline natural waters, and many species in laboratory culture exhibit high rates of growth and photosynthesis only at an alkaline pH (16). Conversely, most blue-green algae are unable to grow or photosynthesize in an acidic environment.

As the HCO_3^- ion is the predominant species of DIC[2] at pH values in the range of 7.0 to 10.0 (5), the capacity of these algae to grow in an alkaline environment suggests that they are capable of assimilating HCO_3^- as a substrate for photosynthetic carbon fixation. Previous studies in this laboratory have shown that the blue-green alga *Coccochloris* is indeed capable of transporting HCO_3^- at an alkaline pH (8, 19, 20). Other reports indicate that the result of this transport is the formation of a large, internal, inorganic carbon pool prior to photosynthetic carbon fixation (2, 19). This study is an examination of the effect of external pH on photosynthesis and the accumulation of inorganic carbon in the blue-green alga *Coccochloris peniocystis* and a hypothesis is proposed to explain the apparent inhibition of photosynthesis at an acid pH.

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[2] Abbreviations: DIC, dissolved inorganic carbon; DMO, 5,5-dimethyloxazolidine-2,4-dione; RuBP, ribulose 1,5-bisphosphate.

MATERIALS AND METHODS

Coccochloris peniocystis Kutz (U.T.E.X. No.: 1548), obtained as an axenic culture from the algal collection at Indiana University, Bloomington, IN, was cultured on air levels of CO_2 and harvested as previously described (18). Photosynthetic rates of algal cell suspensions (5 μg Chl ml^{-1}) in buffer at 30 C were measured as O_2 evolution, at saturating DIC concentrations and a light intensity of 2.5×10^4 μw cm^{-2}, in a temperature-controlled Clark-type O_2 electrode (Hansatech Ltd., Kings Lynn, Norfold, U. K.), calibrated as described previously (10). Before determination of photosynthetic rates and carbon accumulation the cells were preincubated for 10 min in an illuminated water bath at the prescribed pH and aerated under identical conditions as those described for measurement of O_2 evolution. Buffers used were: pH 5.0 to 6.5, 50 mM Mes; pH 7.0 to 8.5, 50 mM Hepes; and pH 9.0 to 11.0, 50 mM glycine-NaOH.

Inorganic Carbon Accumulation. The assay for the accumulation of inorganic carbon utilized the technique of rapid separation of the cells from the incubation medium by centrifugation through a silicone fluid layer (13, 15). To a 400-μl Eppendorf microtube was added 100 μl of an alkaline killing solution (10% methanol in 2 M NaOH), followed by 50 μl silicone fluid (Wacker-Chemie, Munich, F.R.G.). The two layers were compacted by a brief centrifugation and the prepared tubes maintained at 30 C. After preincubation at a known pH, 50 μl of a cell suspension (20 μg Chl ml^{-1}) was layered on top of the compacted silicone fluid and placed in the head of an Eppendorf micro-centrifuge. Illumination of the incubation layer was provided by a slide projector (Leitz-Wetzlar, F.R.G.) giving a light intensity of 3.0×10^4 μw cm^{-2} incident upon the surface. The cell suspension in the tube was illuminated for 30 s before the injection of 10 μl Na$H^{14}CO_3$ (0.5-1.0 μCi μmol^{-1} C). Photosynthesis was stopped after prescribed periods of time by centrifugation of the cells through the silicone fluid into the killing solution. Cell recovery, as measured by Chl content of the pellet averaged 85% after 10 s centrifugation. The tubes were then quickly frozen in dry ice-methanol and the bottom cut off at the silicone fluid-killing solution interface. The killing solution was allowed to thaw, the pellet and solution removed and the tube rinsed with a further 100 μl of 2 M NaOH. An aliquot of the recovered cell suspension was assayed for ^{14}C activity by liquid scintillation spectrometry in 10 ml Bray's solution containing 500 μl ethanolamine. The remaining basic solution (150 μl) was acidified by the addition of 200 μl of 2 M $HClO_4$, the acid-labile ^{14}C removed in a CO_2 stream and the remaining ^{14}C activity counted in ACS scintillation cocktail (10 ml, Amersham-Searle Corp.).

In each experiment the intracellular volume of the cells was determined by equilibration of the cells with ^3H_2O in the aqueous incubation layer for 4.0 min while the volume of the extracellular contaminating fluid was determined by similar equilibration with [^{14}C]sorbitol. From these activities and the measurement of cell recovery the incorporation of C was calculated as a function of total Chl (μmol C mg^{-1}Chl) or cell volume (mM DIC). Total DIC

concentrations in the incubation layer, both unlabeled and radioactive, were determined by a gas chromatographic technique (4). This allowed for the exact measurement of specific radioactivity of the [^{14}C]DIC which can change significantly with increasing pH as a result of invasion of the medium by unlabeled CO_2 from the air.

Determination of Intracellular pH. Intracellular pH of *Coccochloris* in the light and dark was determined using the silicone fluid filtration technique and the partitioning of [^{14}C]DMO or [^{14}C]-methylamine between the cell and the incubation medium as described by other workers (11, 14, 21). Cells used in these experiments were preincubated for 10 min at the appropriate pH as previously described.

Assay of RuBP-carboxylase Activity. Cell-free extracts of *Coccochloris* were prepared by spheroplast lysis as previously described (9). RuBP-dependent fixation of $NaH^{14}CO_3$ into acid-stable products was assayed over the pH range of 6.5 to 8.5 by a method similar to that previously described (6). The cell-free lysate was preincubated in the assay mixture for 10 min at 30 C to ensure complete enzyme activation after which the reaction was initiated by the addition of $NaH^{14}CO_3$ and RuBP.

In all experiments, ^{14}C and ^{3}H activities were determined by liquid scintillation spectrometry using a Tracor-Analytic model 6892 spectrometer (Elk Grove, IL). Chl was determined after extraction in methanol (4).

RESULTS

The rate of photosynthesis of *Coccochloris* as measured by O_2 evolution exhibited a broad optimum over the pH range of 7.0 to 10.0 (Fig. 1). This rate of O_2 evolution was strongly inhibited by lowering the pH below 7.0 and photosynthesis ceased at pH values below 5.25.

Inorganic Carbon Accumulation. The rate of carbon accumulation by the algal cells was measured over the pH range of 5.0 to 10.0 in an attempt to determine whether this process was the rate limiting step in photosynthesis at acid pH. The accumulation of carbon was measured using the silicone fluid technique in which the enzymic reactions are terminated by spinning the cells into a strongly basic killing solution. The ^{14}C activity of cells killed in this manner is a measure of both ^{14}C fixed into organic compounds and the ^{14}C inorganic carbon taken up by the cell but remaining unfixed. The size of these two fractions can be determined by measuring the remaining ^{14}C after acidification and the subtraction of this acid-stable activity from the total ^{14}C incorporated. A typical time course of ^{14}C uptake into the acid-stable and the inorganic pool of *Coccochloris* at pH 8.0 is shown in Figure 2. After a 30 s exposure to [^{14}C]HCO_3^- in the light, the inorganic carbon pool constitutes more than 50% of the total ^{14}C accumulated. A 30-s period of illumination was, therefore, used as the standard time interval for measurement of the inorganic carbon pool at different pH values.

The variation in concentration of the internal inorganic carbon pool of *Coccochloris* after 30 s in the light over the pH range of 5.0 to 10.0 is shown in Figure 3. The total external DIC concentration over this pH range varied from 0.56 to 0.89 mM, as measured by the gas chromatographic technique. This increase in total DIC with increasing pH was a result of the invasion of the buffered cell suspension by atmospheric CO_2 during preincubation, as above pH 7.0 buffers act as CO_2 traps. The results in Figure 3 are corrected for the change in specific radioactivity of the [^{14}C]HCO_3^- resulting from increasing pH. The results dem-

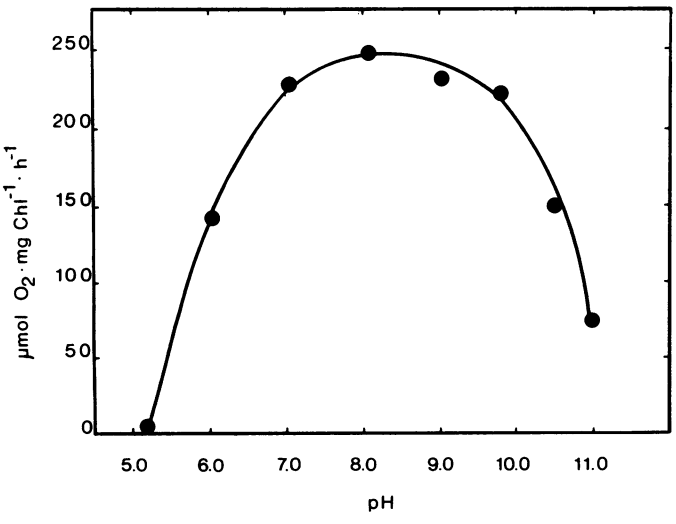

FIG. 1. Effect of external pH on photosynthetic O_2 evolution at an external inorganic C concentration of 5 mM and 30 C.

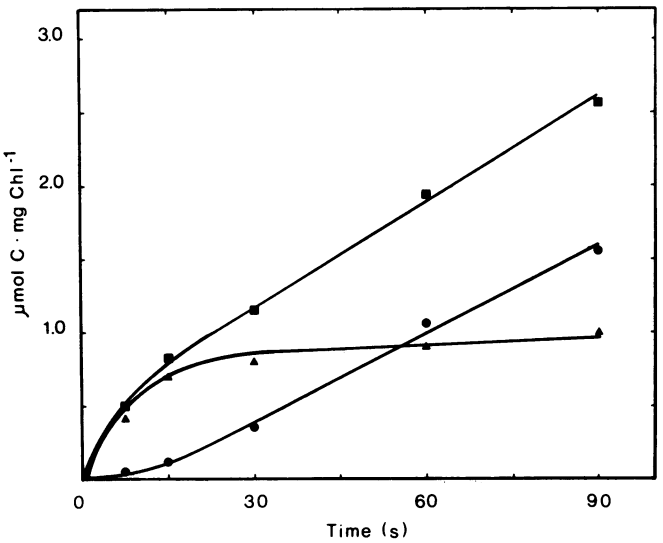

FIG. 2. Time course of inorganic carbon accumulation at pH 8.0, a light intensity of 3.0×10^4 μw cm^{-2} and 30 C. Initial external inorganic C concentration was 207 μM. The figure displays: total carbon accumulated (■), carbon assimilated into acid-stable products (●), and acid-labile inorganic carbon within the cells (▲).

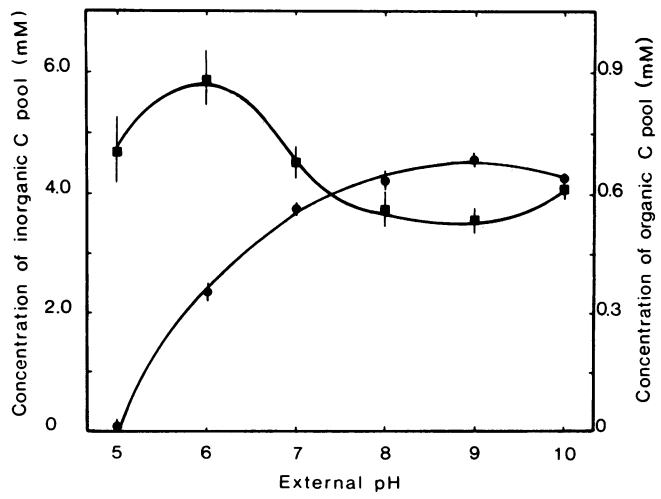

FIG. 3. Effect of external pH on the concentration of the internal acid-labile inorganic carbon pool (■) and the acid-stable (fixed) carbon pool (●) accumulated after 30 s illumination. The external inorganic C concentration was 560 to 890 μM. Vertical bars represent the standard error of the mean.

onstrate little variation in the intracellular inorganic carbon pool over the experimental pH range and at saturating external DIC concentrations. On the other hand, the proportion of carbon fixed by the cells into acid-stable products is substantially reduced below pH 6.5. The ability of *Coccochloris* to accumulate inorganic carbon over the pH range of 5.0 to 8.0 in light and dark is contrasted in Figure 4. At an external DIC concentration of 100 to 125 μM the intracellular concentration was greater than 2.8 mM DIC after 30-s exposure to $NaH^{14}CO_3$ in the light over the entire pH range. In the dark, however, the internal concentration of inorganic carbon after 30-s exposure to ^{14}C was below 2.5 mM at pH 5.0 and dropped rapidly to below 0.5 mM at pH 8.0.

Effect of External pH on Intracellular pH. The intracellular pH of *Coccochloris* was estimated, in the light and dark, using [^{14}C]DMO over the pH range of 5.25 to 7.0 and [^{14}C]methylamine over the pH range of 8.9 to 11.4. At an external concentration of 40 μM, DMO reached equilibrium between the incubation layer and the cell after 4.0 min and no changes in internal concentration or photosynthesis were noted after 30 min incubation. Methylamine at a concentration of 21.0 μM equilibrated much more rapidly, apparently after a 15 s exposure period. The internal concentration remained constant for 3 min after which a steady increase was noted. No effect of methylamine on photosynthesis was observed at 5 times the concentration used for intracellular pH determination and after 30 min exposure. As a result of these time courses, the estimated internal pH was based on the DMO incorporated after 6.0 min and methylamine incorporation after 30 s incubation.

At a pH of 8.0 and at 30 C both compounds are almost totally in the charged form; pKa DMO = 6.28, pKa methylamine = 10.50 (1, 21). As a result of the low concentration of the uncharged species, variable results were obtained and, therefore, the intracellular pH values recorded for this external pH were not used in the determinations in Figure 5 (11). The intracellular pH of *Coccochloris* in the light dropped from 7.56 ± 0.19 at external pH 7.0 to pH 6.58 ± 0.10 at external pH 5.25. Above pH 7.0 the intracellular pH rose gradually to pH 7.74 ± 0.10 at external pH 9.50. Above pH 9.50 the internal pH increased more rapidly, rising to pH 8.31 ± 0.16 at external pH 11.40. In the dark, the intracellular pH over the external pH range of 7.0 to 9.0 responded similarly to changing external pH with most values found to be 0.5 pH units below those recorded for illuminated cells (Fig. 5). Below an external pH of 7.0 and above pH 9.0, the intracellular pH in the dark also appeared to be much more susceptible than in the light to fluctuations in the pH of the surrounding medium.

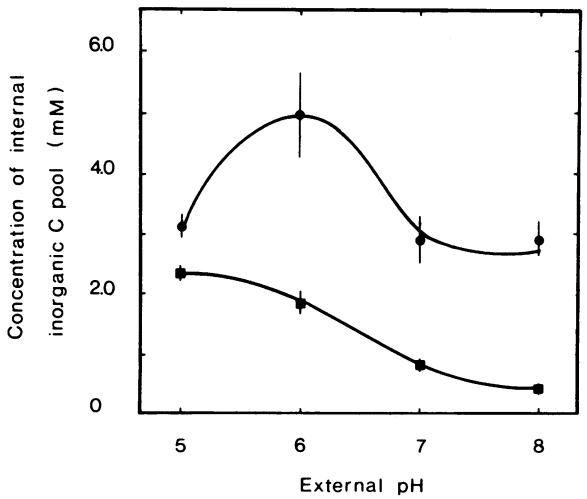

FIG. 4. Effect of external pH on the concentration of the internal inorganic carbon pool accumulated after 30 s in the light (●) and dark (■) at an external inorganic C concentration of 100 to 125 μM. Vertical bars represent the standard error of the mean.

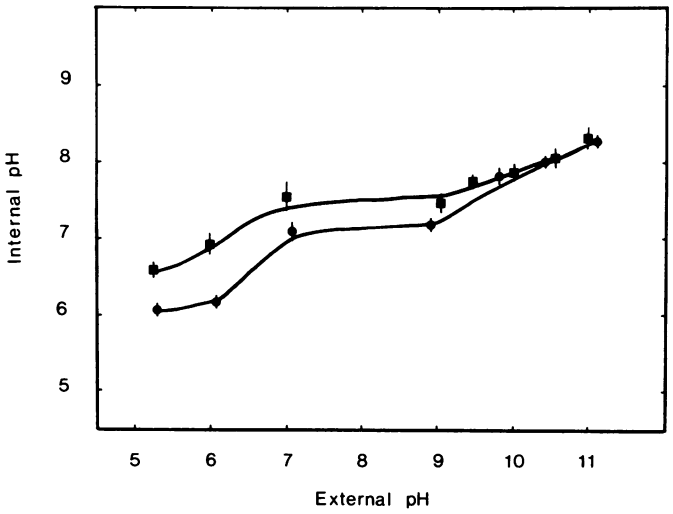

FIG. 5. Effect of external pH on the intracellular pH in the light (■) and in the dark (●). Internal pH was determined after equilibration of [^{14}C]DMO (40 μM) over the external pH range of 5.0 to 7.5, and [^{14}C]-methylamine (21.0 μM) over the pH range of 8.5 to 11.5. Inorganic C concentration was 700 to 950 μM. Vertical bars represent the standard error of the mean.

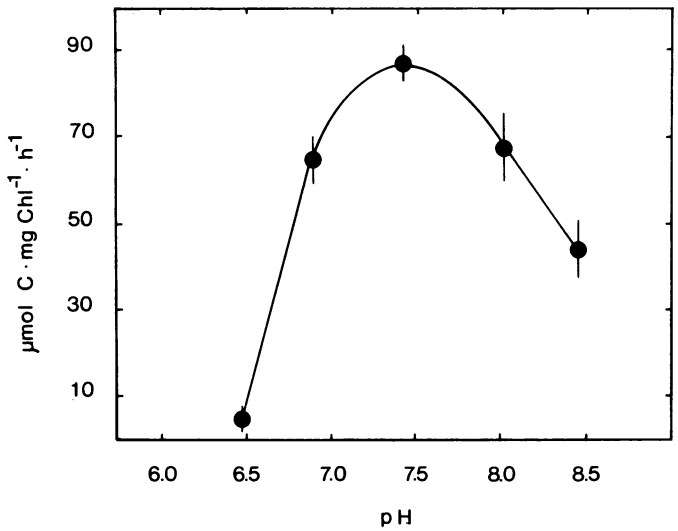

FIG. 6. Effect of pH on the activity of RuBP-carboxylase in cell-free preparations of *Coccochloris*. Vertical bars represent the standard error of the mean.

Effect of pH on RuBP-carboxylase. The activity of the enzyme RuBP-carboxylase in a cell-free preparation, obtained by the osmotic lysis of *Coccochloris* spheroplasts, was examined with respect to pH. The rate of formation of RuBP-dependent acid-stable products over the pH range of 6.5 to 8.5 is shown in Figure 6. RuBP-carboxylase exhibited optimal activity at pH 7.5 to 7.8 but was inhibited substantially below pH 6.8 and found to be essentially inactive at pH 6.5. Above pH 8.0 the activity of RuBP-carboxylase was reduced with the degree of inhibition approaching 50% at pH 8.5.

DISCUSSION

The photosynthetic capacity of the blue-green alga *Coccochloris* is radically affected by the pH of the external medium (Fig. 1). The variation in the rate of O_2 evolution at different external pH

values may indicate an inability of the alga to maintain a constant internal pH. The data show (Fig. 5) that *Coccochloris* is able to regulate the internal pH adequately in the light over the external pH range of 7.0 to 10.0, which corresponds to the optimal pH range for photosynthesis. The estimates of internal pH over this external pH range, both in the light and the dark, are similar to those determined for another unicellular blue-green alga *Anacystis nidulans* (12). At pH values below 7.0 and above 9.5, however, the intracellular pH of *Coccochloris* in the light is significantly influenced by the external pH and it is over these external pH ranges that the rate of photosynthesis is substantially reduced, particularly below pH 7.0. The intracellular pH in the dark is affected to a greater extent by the external pH than that in the light. The cells in the dark are able to maintain a constant intracellular pH only over a small external pH range (pH 7.0 to 9.0) and exhibit large internal pH fluctuations above and below this pH range (Fig. 5).

Inasmuch as the internal pH may be a factor which determines the uptake, across the cell membrane, of inorganic carbon during photosynthesis, the capacity of *Coccochloris* to accumulate carbon was examined over a wide pH range.

The carbon accumulated by this alga was found in two distinct fractions: one fraction consisted of acid-stable products of photosynthesis whereas the other was acid-labile and represents an intracellular inorganic carbon pool (Fig. 2). This pool, after very short periods of incubation in the light constitutes a major portion of the accumulated carbon. The size of the inorganic carbon pool after a fixed period of illumination (30 s) appears to be constant over a wide pH range indicating that the capacity for carbon accumulation is not reduced at external pH values which are inhibitory for O_2 evolution (Fig. 3).

At an alkaline pH where the intracellular pH is less than the external pH, the cell must rely upon active transport of HCO_3^- and not a passive flux of CO_2 if inorganic carbon accumulation is to occur (3). In a more acidic environment, where the internal pH exceeds that of the surrounding medium, and where CO_2 comprises a major portion of the total external DIC, it is thought that carbon accumulation is accomplished by the passive movement of CO_2 along a pH gradient into the cell or chloroplast (3, 22). In the dark, therefore, the cell is able to maintain a large, inorganic carbon pool only at acid pH values presumably because of the large pH gradient across the cell membrane (Fig. 4). For example, in medium at pH 5.0, the ΔpH in the dark is approximately +1.0 pH units, whereas at pH 7.0 the estimated ΔpH is 0.1 units (Fig. 5). As the difference in pH between the cell interior and the surrounding medium becomes progressively smaller and is subsequently reversed at alkaline pH values above the intracellular pH, the alga in the light maintains the internal inorganic carbon pool by active transport of HCO_3^-. This is consistent with the large light/dark difference in the inorganic carbon pool observed at external pH values of 7.0 and 8.0 (Fig. 4). This does not preclude the possibility of some active HCO_3^- transport occurring at acid pH values in the light since a significant portion (30.8%) of the available DIC in the medium is in the form of HCO_3^- (5). Although a pH gradient of +0.9 pH units exists in the light at an external pH of 6.0, it is insufficient to account for the formation, by simple diffusion, of the large internal inorganic carbon pool. The additive effect of CO_2 diffusion along a pH gradient and HCO_3^- transport may account for the size of the internal inorganic carbon pool observed at pH 6.0 in the light (Fig. 4).

Although no marked change occurred in the size of the internal inorganic carbon pool over the pH range examined, there was a significant decrease in the portion of the total accumulated carbon found in the acid-stable fraction at pH 5.0 and 6.0. This result correlates well with the decrease in both internal pH and rate of O_2 evolution at these same pH values. As the principal pathway of carbon fixation in this alga is known to be mediated by the enzyme RuBP-carboxylase (7), an examination of its activity *in vitro* over a pH range of 6.5 to 8.5 was performed (Fig. 6). This range simulated the estimated intracellular pH of the alga over the external pH range of 5.25 to 11.25. The data suggest that at an external pH of 5.25 where rates of O_2 evolution and carbon fixation are negligible, the intracellular pH of *Coccochloris* has been lowered to such an extent that the activity of RuBP-carboxylase is minimal. When the external pH is raised above this point, the intracellular pH is increased and the activity of RuBP-carboxylase rises until the optimal pH of 7.5 to 7.8 is obtained. At extreme alkaline pH values where photosynthetic O_2 evolution is inhibited, several factors may contribute to the reduction in photosynthesis in addition to the observed decrease in RuBP-carboxylase activity (17). A similar response of RuBP-carboxylase activity with pH has recently been noted for the purified enzyme isolated from the blue-green alga *Anabaena variabilis* (2).

In summary, at most alkaline pH values, where photosynthesis of the blue-green alga *Coccochloris* is optimal, the intracellular pH was found to be lower than that of the surrounding medium. The resultant pH gradient across the plasmalemma precludes any accumulation of inorganic carbon simply by the inward diffusion of CO_2. The internal inorganic carbon pool formed in the alga, in the light and at these alkaline pH values must, therefore, be the product of active HCO_3^- transport. This paper provides additional evidence that HCO_3^- transport does occur in *Coccochloris* (8, 19, 20). The inability of this procaryotic organism to photosynthesize optimally below pH 7.0 is not the result of carbon limitation but may be explained by the inactivation of RuBP-carboxylase at lowered internal pH values.

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