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# The Effect of pH on the Products of Photosynthesis in $^{14}CO_2$ by Chloroplast Preparations from *Acetabularia mediterranea*[1]

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## ABSTRACT

**The effect of pH on the pathways of carbon in photosynthesis was examined in chloroplast preparations from *Acetabularia mediterranea*. The flow of carbon into a number of photosynthetic intermediates, particularly sucrose, glycine, serine, glycolate, and the insoluble fraction, was strongly influenced by pH. At higher pH a much larger portion of the $^{14}C$ entered intermediates of the glycolate pathway. Although maximal apparent photosynthesis occurred at pH 7.6 to 7.7, cytoplasmic pH was found to be 8.0 to 8.4, using indicators. The pattern of distribution of $^{14}C$ in intermediates of whole cells was closest to that in chloroplasts at the higher pH range.**

A number of studies have established the pH for the maximal rate of carbon fixation in cells (15) and in isolated chloroplasts (7, 9, 10). Early experiments of Calvin *et al.* (6) indicated that *Chlorella* cells could withstand major changes in pH of the medium, and that these changes resulted in different patterns of $CO_2$ fixation. The incorporation of $^{14}CO_2$ into malate and sucrose was strongly dependent on pH. Ouellet and Benson (15) examined the photosynthetic and dark fixation of $^{14}CO_2$ in *Scenedesmus* over a pH range from 1.6 to 11.4 and found that high pH favored $^{14}C$ incorporation into four-carbon compounds, while lower pH favored fixation into three-carbon compounds, as well as sucrose and polysaccharides. Orth *et al.* (14), using *Chlorella* and *Scenedesmus*, found substantially higher incorporation of $CO_2$ into serine, glycine, and glycolate at pH 8.8 than at pH 7.0.

We are not aware, however, of any studies showing the effect of pH on either the intermediates of photosynthesis or the pathway of carbon in isolated chloroplasts. Preliminary work in this laboratory with chloroplast preparations from *Acetabularia mediterranea* suggested that pH affects the pattern of photosynthetic incorporation of $^{14}C$ into most intermediates of the glycolate pathway and the photosynthetic carbon reduction cycle (F. Winkenbach and R. G. S. Bidwell, unpublished). This paper describes the results of an investigation of the effect of pH on the incorporation of $^{14}CO_2$ into photosynthetic intermediates in these chloroplast preparations.

## MATERIALS AND METHODS

**Chloroplast Preparation.** Cells of *Acetabularia mediterranea* were maintained in laboratory culture, and chloroplasts were prepared by methods previously described (4, 16). Chloroplast preparations were made at 4 C. The H, W, and A media were identical to those of Bidwell *et al.* (4), except that the W medium was adjusted to pH 7.5 prior to use, and the concentration of TES[3] or tris in the A medium was increased from 5 to 50 mM. Tests showed that this concentration of the buffers affected neither the rate of photosynthesis nor the distribution of $^{14}C$ among products. Tris-buffered A medium was used for the pH ranges above 8.0 and TES-buffered A medium below pH 8.0. The pH of the A medium was adjusted to the appropriate experimental value by addition of either 1 N HCl or 1 N KOH. The pH of suspensions was monitored and remained essentially unchanged during experiments.

Fresh cells (1 g) were blotted to remove excess culture solution, then placed in 5 ml of H medium and rapidly chopped into small pieces with a razor blade. The mixture was centrifuged at 600*g* for 5 min. The supernatant fraction was discarded and the pellet was transferred to a Teflon homogenizer with 5 ml of W medium and homogenized slowly by hand in an ice bath for 30 sec. The extract was filtered through two layers of 173-mesh bolting silk and centrifuged at 600*g* for 5 min. The pellet was resuspended in A medium as required. The chloroplast preparations were examined by phase contrast microscopy and found to have a low level of cytoplasmic contamination, similar to earlier preparations (4). Chlorophyll was determined by the method of Arnon (2).

**Measurement of Photosynthetic Rates.** The reaction mixture contained 2.15 ml of A medium of appropriate pH and 0.1 ml of chloroplast suspension (approximately 100 $\mu$g of chlorophyll). The reaction mixture was briefly flushed with $N_2$ prior to addition of the chloroplast suspension to reduce $O_2$ concentration to approximately 10 to 15% of air saturation. Warburg analysis showed that total carbon content of the medium was not affected by this treatment. The reaction vessel (YSI electrode cuvette) was maintained at 20 C in a constant temperature bath and illuminated by a 500-w water-screened incandescent lamp. The light intensity was reduced by a 20% neutral density filter to give an intensity of $2.5 \times 10^5$ ergs $cm^{-2}sec^{-1}$ in the reaction vessel. The reaction mixture was preilluminated for 3 min before addition of $Na_2{}^{14}CO_3$.

**Oxygen Evolution.** $O_2$ concentration was determined with a Clark-type $O_2$ electrode (YSI model 5331). The $O_2$ electrode

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[3] Abbreviations: PGA: 3-phosphoglyceric acid; HMP: hexose monophosphates; TES: *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

was calibrated by adding catalase to A medium and adding known volumes of a standard solution of $H_2O_2$ (11).

**Carbon Dioxide Fixation.** Total carbon fixed was measured, after injecting $^{14}C$-carbonate solution of known specific radioactivity into the reaction mixture, by withdrawing 10-$\mu$l aliquots with a Hamilton syringe at intervals and applying these to Millipore filters (12). Radioactive carbonate was removed by placing the filter in a desiccator over 6 N HCl for 1 hr. The filter was then placed in a counting vial with 10 ml of scintillation solution [4 g 2,5-diphenyloxazole (PPO) and 0.5 g 1,4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP) per liter dioxane:anisole:dimethoxyethane (6:1:1 by volume)].

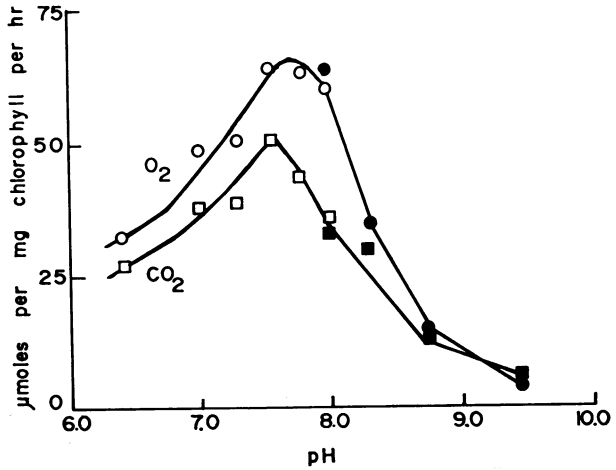


FIG. 1. Influence of pH on carbon fixation and $O_2$ evolution by *Acetabularia* chloroplast preparations. Medium was buffered with TES (open symbols) or tris (closed symbols). $O_2$ concentration was 2–4%.

**Chromatography and Radioautography.** After 10 min of photosynthesis in $^{14}CO_2$, a 25-$\mu$l aliquot was removed from the reaction vessel with a Hamilton syringe and spotted onto Whatman 3MM chromatography paper. The origin was immediately treated with hot ethanol vapors to inactivate enzymes (3, 5). The photosynthetic products formed were separated by two-dimensional chromatography (3) and radioautographed using Kodak No-Screen x-ray film. Radioactivity in the spots was determined in a Nuclear-Chicago gas flow counter (5). Glucose was separated from serine and glycine after chromatography by passing material eluted from the chromatogram through Dowex 50W-X8 resin.

**Specific Radioactivity Calculations.** The total concentration of carbon present in solution in the A medium as $CO_2$, bicarbonate, and carbonate (hereafter called "total carbon") was determined experimentally by Warburg manometry. The medium used at all pH values was equilibrated with air for 20 hr before the determinations were made. The final specific radioactivity of carbon in the medium at each pH was calculated from the known specific radioactivity of added $^{14}C$ and the amount of total carbon in the A medium. Values for radioactivity fixed could then be used to determine the amounts of carbon fixed photosynthetically.

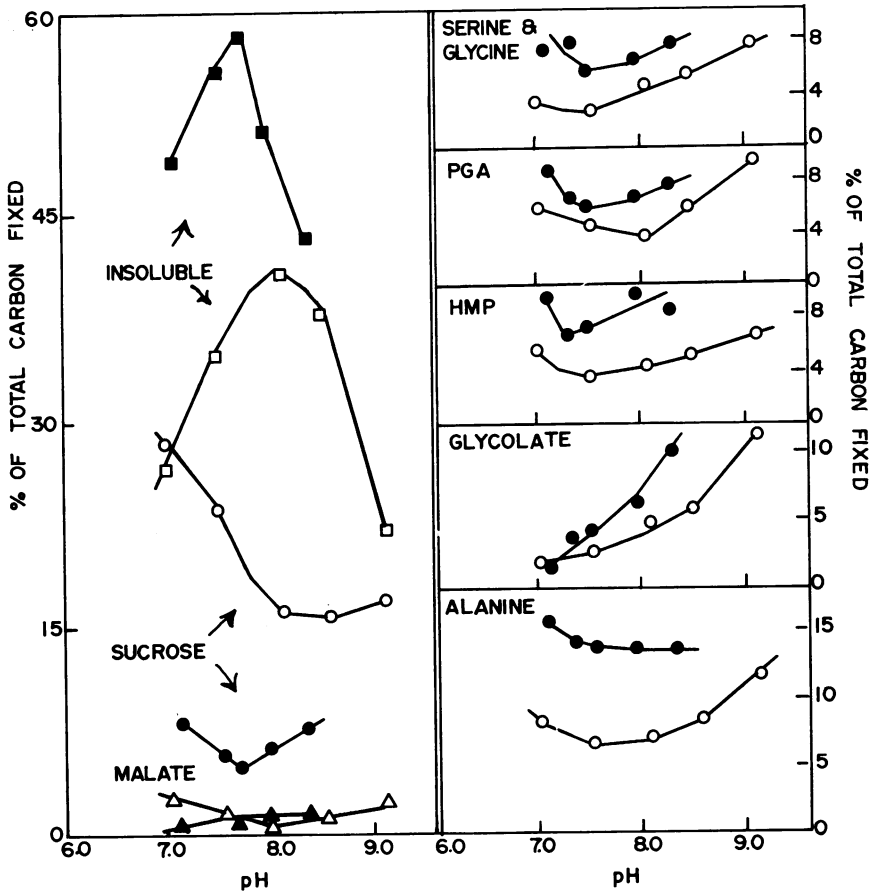


FIG. 2. Distribution of radioactivity in preparations of *Acetabularia* chloroplasts after photosynthesis with $^{14}C$ at different pH. Reaction mixtures contained chloroplasts (approximately 120 $\mu$g chlorophyll) in 400 $\mu$l of A medium and were stirred in a vessel open to the air for 12 min in experiment 1 (open symbols) and 10 min in experiment 2 (closed symbols).

**Carbon Dioxide Fixation in Intact Cells.** Three large *Acetabularia* cells without caps were placed in the reaction vessel with 2.2 ml of artificial sea water at pH 7.6, using the same light and temperature conditions as above. $Na_2{}^{14}CO_3$ was injected into the reaction vessel. After 10 min of photosynthesis in $^{14}CO_2$ the cells were removed, the cell tips were cut off with a scalpel, and the contents were immediately extruded onto Whatman 3MM chromatography paper and inactivated in hot ethanol vapors (3).

## RESULTS

Preliminary experiments showed that chloroplast preparations from *Acetabularia* had maximal photosynthetic rates, as measured by both $O_2$ evolution and $CO_2$ uptake, at pH 7.6 to 7.7 (Fig. 1). The difference between $O_2$ and $CO_2$ measurements of photosynthetic rate has frequently been observed in experiments of short duration (17, and unpublished data from this laboratory). It decreases and disappears after prolonged photosynthesis. The reason for this early imbalance is not clear.

The results of two preliminary experiments in air (Fig. 2) showed that the distribution of $^{14}CO_2$ among photosynthetic intermediates was significantly affected by pH. The differences between the two experiments are probably due to the normal variation among different batches of cells and preparations, and to the difference in sampling time. The percentage of total $^{14}CO_2$ entering most of the intermediates of photosynthesis was strongly affected by pH (Fig. 2). Of particular interest is the increasing incorporation of $^{14}C$ into serine-glycine and glycolate at higher pH, and also the significant effect of pH on sucrose and the insoluble fraction.

There remained the possibility that the pH effects might be due to differences in carbon concentrations in the media. An experiment was performed to test this possibility using the closed reaction vessel described. The total radioactivity added and actual pH (7.2) of the medium were held constant, and $Na_2{}^{12}CO_3$ was added to the reaction vessel to give total carbon concentrations equivalent to those found at pH values from 7.2 to 9.0. It is apparent (Table I) that the difference in carbon concentration was not responsible for the pH effect observed. The distribution of $^{14}C$ among the products of photosynthesis was not affected by the total $^{14}C$ uptake or by the concentration of total carbon in the medium. Also, under the conditions of these experiments, the pools of Calvin cycle intermediates and glycolate become saturated within 3 to 7 min, and differences in radioactivity of end products are well established before 10 min (5, and unpublished data). These facts indicate that the pH effect is not caused by variations in saturation (*i.e.*, specific radioactivity) of pools resulting from rate-dependent kinetic differences among the samples.

A new experiment was performed under the more precisely controlled conditions described. The reaction cuvette allows virtually no gaseous exchange and thus represents a closed system in which it is possible to control the content of carbon in solution. Oxygen concentration was held between 2 and 5%. It is evident that not only the total fixation but also the distribution of carbon between soluble and insoluble fractions was markedly affected by pH (Fig. 3). The insoluble fraction, which accounted for approximately two-thirds of total carbon fixed at pH 7.5, decreased to a much lower proportion at lower pH and to almost nothing at higher values. It appears that the effect of pH on the total carbon in the soluble fraction was not very great. However, there was a significant effect of pH on most of the individual photosynthetic intermediates which comprise the soluble fraction (Fig. 4). In particular, the proportion of $^{14}C$ entering compounds of the glycolate pathway (glycine, serine, and glycolate), as well as numbers of other compounds not normally considered to be photosynthetic intermediates, increased greatly at higher pH. Sucrose, on the other hand, contained a much higher proportion of the fixed carbon at lower pH values. The effect of pH at 20% $O_2$ (Fig. 2) is essentially similar to its effect at lower concentrations of $O_2$ (Fig. 4).

Since pH has a significant influence on the distribution of

Table I. *Effect of Total Carbon Concentration in Medium upon Distribution of $^{14}C$ among Products of Chloroplast Photosynthesis*

Values are means of two replicates. Total carbon concentrations correspond to air-equilibrium concentrations in the medium at the pH values shown. Actual pH of the medium was 7.2 and the period of photosynthesis was 10 min.

| | Total Carbon Concentration | | | |
|---|---|---|---|---|
| | 0.1 mM | 0.73 mM | 3.2 mM | 13.8 mM |
| | Simulated pH | | | |
| | 7.2 | 7.8 | 8.4 | 9.0 |
| | $^{14}C$ in analyzed compounds | | | |
| | *% of total fixed* | | | |
| Insoluble | 54.5 | 49.6 | 54.0 | 51.3 |
| Sugar diphosphates | 0.7 | 0.4 | 0.4 | 0.6 |
| HMP | 0.7 | 1.4 | 1.4 | 1.4 |
| PGA | 7.2 | 4.7 | 4.5 | 6.1 |
| Triose phosphate | 0.1 | 0.1 | Trace | Trace |
| Sucrose | 26.6 | 35.1 | 31.5 | 31.3 |
| Glycolate | Trace | Trace | Trace | Trace |
| Aspartate | 3.9 | 2.8 | 2.7 | 2.7 |
| Glutamate | 1.0 | 0.9 | 0.9 | 1.0 |
| Serine | 1.3 | 1.0 | 1.0 | 1.1 |
| Glycine | 0.1 | 0.1 | 0.2 | 0.2 |
| Alanine | 2.0 | 2.0 | 2.0 | 2.2 |
| Malate | 0.7 | 0.6 | 0.7 | 0.9 |
| Unidentified | 1.1 | 1.3 | 0.7 | 1.1 |
| Total $^{14}C$ fixed, μmoles/mg chlorophyll | 2.8 | 4.0 | 7.7 | 10.4 |

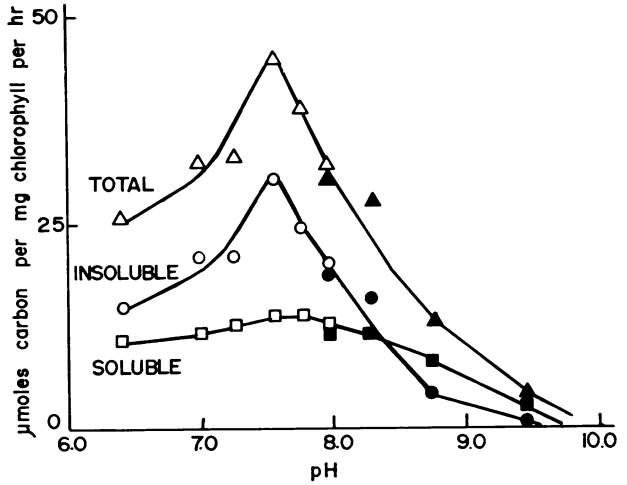


FIG. 3. Effect of pH on total carbon fixed into soluble and insoluble fractions during photosynthesis for 10 min in $^{14}C$ by preparations of *Acetabularia* chloroplasts. Medium was buffered with TES (open symbols) or tris (closed symbols). $O_2$ content was 2–5%.

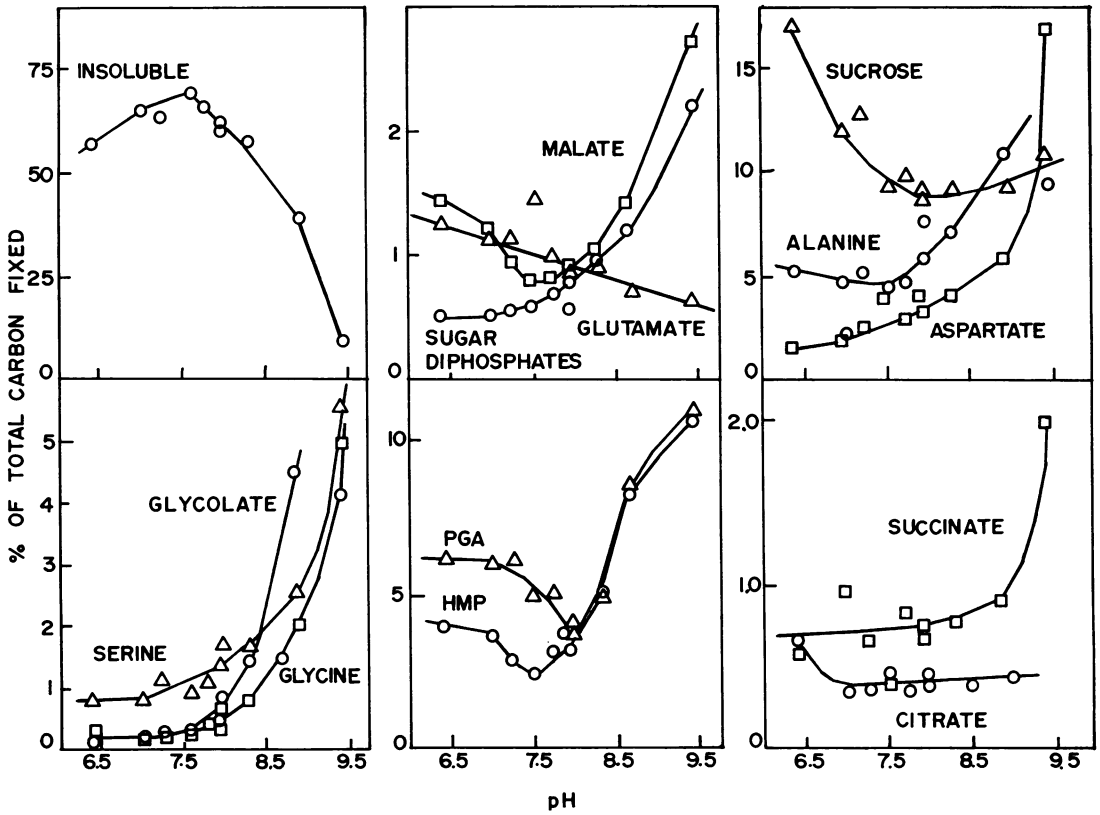


FIG. 4. Effect of pH on the distribution of radioactivity among products of photosynthesis for 10 min in $^{14}C$ by preparations of *Acetabularia* chloroplasts. $O_2$ content was 2–5%.

Table II. *Percentage Incorporation of $^{14}C$ into Whole Cells of Acetabularia*

The values represent the mean of four replicates of three mature cells. Experimental conditions were identical with those used for chloroplasts in Figure 4.

| Compounds Analyzed | Incorporation | Standard Error of Mean | Predicted pH (from Fig. 4) |
|---|---|---|---|
| | % total $^{14}C$ | | |
| Insoluble | 61.7 | 1.5 | 8.0 |
| Sugar diphosphates | 3.3 | 0.23 | 9.5 |
| HMP | 1.1 | 0.15 | |
| PGA | 11.1 | 1.18 | 9.2 |
| Sucrose | 8.0 | 0.75 | 8.2 |
| Glycolate | 0.9 | 0.11 | 8.0 |
| Glycine | 2.1 | 0.33 | 8.7 |
| Serine | 1.4 | 0.13 | 7.7 |
| Alanine | 3.5 | 0.70 | 7.5 |
| Other | 7.9 | 1.36 | |
| Average of predicted pH values | | | 8.3 |

$^{14}C$ fixed during photosynthesis in chloroplasts *in vitro*, it appears possible that cytoplasmic pH may influence the pathway of carbon during photosynthesis *in vivo*. We therefore attempted to measure the pH of cytoplasm of *Acetabularia* cells, uncontaminated by the strongly acidic vacuolar sap. Cells were suspended vertically by their rhizoids in centrifuge tubes containing artificial sea water and centrifuged at 500*g* for 10 min. The cytoplasm which lodged in the tip of the cell was tied off with thin nylon thread and the cell wall ruptured under a low power microscope. The pH indicators phenolphthalein and thymol blue, prepared in 0.6 M mannitol to give isotonic solutions, were used to estimate the pH of the cytoplasm as it flowed out. Approximately eight different estimations were made with each indicator. The cytoplasmic pH as measured by these indicators was between 8.0 and 8.4. Dual-Tint pH indicator papers (J. T. Baker and Co.) were also used and gave a value of approximately pH 8.2. The distribution of $^{14}C$ among intermediates of whole cell photosynthesis under identical conditions (Table II) was compared with that of chloroplasts (Fig. 4). pH values of the cytoplasm predicted from the chloroplast data varied from 7.5 to 9.5 and gave an average indication of 8.3 for cytoplasmic pH. This very rough estimation agrees well with the measured value.

## DISCUSSION

The data in Figures 2 to 4 show that pH exerts a significant effect on the flow of carbon into photosynthetic intermediates in *Acetabularia* chloroplasts. Furthermore this effect can be ascribed to pH *per se*, and not to the effect of differing carbon concentrations in the medium resulting from pH changes (Table I). One of the most significant effects of pH is on the $^{14}C$ incorporation into the insoluble fraction. This fraction comprises compounds immobile in the chromatographic solvent system, probably mostly storage and structural polymers, the synthesis of which is thus markedly affected by pH. Therefore the existence of a pH-sensitive mechanism is implied, which is somehow capable of proportioning carbon between the pathways leading to soluble and insoluble products. Most of the compounds in the soluble fraction are also significantly affected by pH. The effect on glycine, serine, and glycolate is particularly marked and indicates a much more significant role

for the glycolate pathway at higher pH (14). The mechanism whereby pH affects the distribution of photosynthetic carbon cannot be determined from these experiments.

Experiments with isolated chloroplasts usually have been done in the past at pH of approximately 7.2 to 7.6 (3, 4, 8, 13). The maximal rate of apparent photosynthesis occurs at about pH 7.7 as measured by $O_2$ evolution in *Acetabularia* chloroplasts (Fig. 1), or by $^{14}C$ fixation in spinach or *Acetabularia* chloroplasts (9, 10, Fig. 1). However, this pH region may not necessarily be optimal for some of the photosynthetic reactions, nor physiologically normal. Our experiments with pH indicators and $^{14}CO_2$ fixation using *Acetabularia* cells indicate that the cytoplasmic pH is 8.0 to 8.4. It may be significant that Allen *et al.* (1) have shown that photosynthetic phosphorylation in spinach chloroplast fragments has a sharp optimum at pH 8.3.

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