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The Role of Glycolic Acid Metabolism in Opening of Leaf Stomata [1]

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The initial observation (23) that α-hydroxysulfonates, which are effective competitive inhibitors of glycolate oxidase (20), prevent the opening of stomata in the light stimulated further experiments on the physiology of stomatal movement (15). It seemed reasonable to suspect that the enzymic oxidation of glycolic acid was connected with the process of stomatal opening, and hence that any interference with either the synthesis or further oxidative metabolism of this substrate might also result in an inhibition of stomatal opening.

In a previous report (15), we presented evidence, obtained from a standard leaf disk assay of stomatal closure, that the processes of opening and closing are controlled by different and independent mechanisms. By use of biochemical inhibitors as well as by other means, it was found possible to inhibit opening and thereby induce closing. In other related investigations, the effect of the chemical control of stomatal opening on the rates of transpiration and photosynthesis have been investigated in the laboratory, the greenhouse, and outdoors (10, 11, 14, 25, 27, 28).

Further experiments have now been carried out on the mechanism of action of α-hydroxysulfonates on stomatal movement, and of the effect of the concentrations of CO_2 and O_2 in the atmosphere surrounding the leaf tissue on the synthesis of glycolic acid and on stomatal movement. The results clearly demonstrate the close relation between the synthesis and metabolism of glycolic acid and the process of stomatal opening that takes place in the light. A preliminary account of these experiments has been published (24).

Materials and Methods

Tobacco leaves (*Nicotiana tabacum*) were kept in the dark for an hour before the start of the experi-

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ments so that the stomata were initially closed. The standard assay of stomatal closure (23) was performed with leaf disks 1.6 cm in diameter that were cut with a sharp punch. The disks were taken from a single leaf in each experiment, and pairs were floated on water so that hydration was never a limiting factor. At the end of the experiment, carried out at 30° in the light (about 2,000 ft-c provided by flood lamps), silicone rubber impressions were cast of the lower epidermis (9). Films of cellulose acetate were then prepared from the impressions, and the mean stomatal width was determined by measurement under the microscope of 50 apertures from each of the pair of leaf disks.

For determination of the glycolic acid concentration in the tissue, leaf disks were quickly transferred to boiling water and maintained at this temperature for 3 minutes. The killed tissue was homogenized and the extract placed on a Dowex 1-acetate anion exchange column. The fraction containing the glycolic acid was collected by elution of the column with 4 N acetic acid (21), and the concentration of glycolic acid was determined by the colorimetric method of Calkins (4). Excellent recoveries of added glycolic acid are obtained by this procedure (21).

In experiments where the gas phase surrounding the disks was altered, disks were floated on 2.5 ml of water or on a solution of inhibitor or metabolite in 25-ml Erlenmeyer flasks which were closed with rubber serum stoppers (15). The gas, supplied from cylinders, was first bubbled through water, and then was passed continuously through the flasks at a rate of several flask volumes per minute.

The substrates used were the highest grade commercially available. Phosphorylated compounds were first passed through a Dowex-50 cation exchange column in the H^+ form, and were then neutralized to about pH 6.0 with sodium bicarbonate.

Experimental Results

Relation between Stomatal Opening and Glycolic Acid Accumulation. The α-hydroxysulfonates are analogues of glycolic acid and are effective and highly specific competitive inhibitors of purified glycolate oxidase obtained from spinach leaves (20). It was previously shown that when tobacco leaves, or leaves of several other species, were placed with their bases in a solution of one of these inhibitors in the light, a rapid and extensive accumulation of glycolic acid occurred in the tissue (21, 22). High rates of accumulation of glycolic acid have now also been observed in tobacco leaf disks floating on solutions of the especially effective inhibitor α-hydroxy-2-pyridinemethanesulfonic acid in the light under conditions similar to those used for the stomatal assay.

Figure 1 illustrates an experiment in which the effect of inhibitor concentration on the extent of glycolic acid accumulation in leaf disks was compared with the effect of the same concentrations of the α-

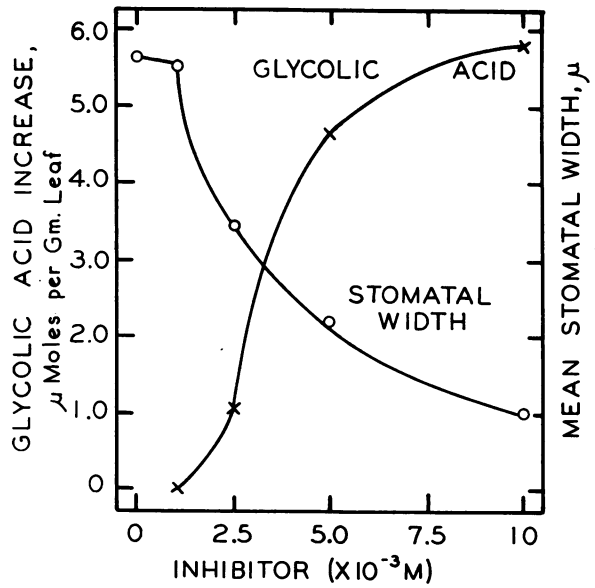

FIG. 1. Effect of concentration of α-hydroxy-2-pyridinemethanesulfonic acid on glycolic acid synthesis and stomatal opening. Disks were floated on the concentration of inhibitor shown for determination of glycolic acid (6 disks for each analysis) or for measurement of stomatal aperture (2 disks) after 120 minutes in the light.

hydroxysulfonate on the degree of stomatal opening under the same conditions. An excellent correlation was found between the effectiveness of a given concentration of inhibitor on the relative inhibition of glycolate oxidase in vivo and the extent of prevention of stomatal opening. These data are therefore consistent with the hypothesis that the oxidation of glycolic acid is essential for the stomatal opening process, and that α-hydroxysulfonates prevent stomatal opening by interfering with the glycolate oxidase reaction.

Reversal by Glycolic Acid of Stomatal Closure Induced by α-Hydroxysulfonate. The competitive nature of the inhibition of glycolate oxidase by α-hydroxysulfonates was clearly established with experiments on purified glycolate oxidase (20). From the form of the Lineweaver-Burk equation derived by Wilson (19),

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i}\left(\frac{[I]}{K_s + [S]}\right)$$

if inhibition is competitive, a plot of the ratio of the uninhibited to the inhibited rate of reaction against different concentrations of inhibitor, [I], should give straight lines with an intercept of 1 and with slopes inversely proportional to the substrate concentration, $[S]$.

In the experiment illustrated in figure 2, the ratio of the mean stomatal width, the steady state between the rates of opening and closing (15), in the absence

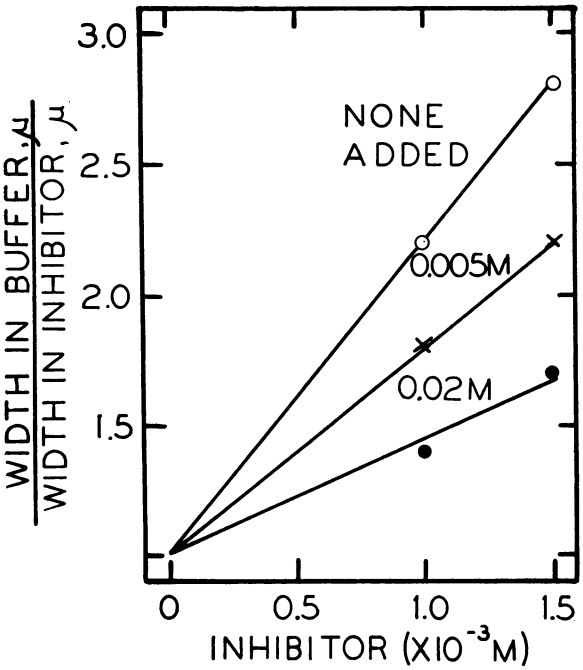

FIG. 2. Competitive inhibition of sodium 1-hydroxy-1-decanesulfonate with glycolic acid on stomatal opening. Leaf disks were floated on 0.005 M sodium tartrate buffer at pH 4.6 in the light for 120 minutes. The disks were then removed, blotted, and returned to solutions of the inhibitor or inhibitor with glycolate at the concentrations shown for 90 minutes more in the light. The mean stomatal aperture of the disks in buffer throughout was 9.4μ.

of inhibitor to the stomatal width in the presence of sodium 1-hydroxy-1-decanesulfonate has been plotted against inhibitor concentration. The addition of increasing amounts of glycolic acid overcomes the stomatal closure brought about by the α-hydroxysulfonate, and in a manner that conforms to the above equation describing competitive inhibition. These results correspond to the enzyme experiments and further support the view that the inhibitors induce stomatal closure because of their interference with the metabolism of glycolic acid in the tissue.

Specificity of Glycolic Acid in Reversing Stomatal Closure by Several Inhibitors. The stomatal closure brought about by α-hydroxydecanesulfonate can be reversed by supplying glycolic acid to the leaf disks and the effect appears to be specific. Neither glucose, potassium chloride, nor malate increased the ability of the stomata to open significantly (table I, experiment 1) when tested under the same conditions. On the other hand, glycolic acid did not enhance stomatal opening in the presence of inhibitors such as azide or phenylmercuric acetate (table I, experiment 2). Among the many experiments of this kind that were carried out, a partial reversal of azide inhibition by glycolic acid was observed from time to time, but the inability of glycolic acid to reverse the stomatal closure brought about by phenylmercuric acetate was a constant finding. These experiments also support the hypothesis that stomatal closure induced by α-hydroxysulfonates is the direct result of an interference with the normal metabolism of glycolic acid, and also suggest that other inhibitors block the opening process at some different stage in the mechanism (24).

Requirement of O_2 for Stomatal Opening and Glycolate Synthesis. We have previously shown that under anaerobic conditions, the rate of stomatal opening in leaf disks in the light is greatly inhibited (15). Bassham and Kirk (2) and Tanner et al. (13) have reported that an external supply of O_2 stimulates glycolic acid synthesis in *Chlorella*, although quantitative data were not provided. With use of an effective α-hydroxysulfonate to inhibit oxidation of the glycolic acid produced in the leaf disks, the accumulation of glycolic acid in the light was compared in an atmosphere of air and in N_2 con-

Table I. *Attempted Reversal of Stomatal Closing by Inhibitors with Various Substrates*

Pairs of tobacco leaf disks were floated on 0.005 M sodium tartrate buffer at pH 4.5 for 90 minutes in light. The disks were then removed, quickly blotted, and returned to the solutions indicated. The solutions were made up in buffer. The disks remained for an additional 90 minutes in the light before silicone impressions were prepared. In experiment 1 the final concentration of α-hydroxydecanesulfonate was 1×10^{-3} M and of the other solutes 2×10^{-2} M. In experiment 2, the final concentrations were α-hydroxydecanesulfonate, 1.5×10^{-3} M; azide, 1×10^{-4} M; phenylmercuric acetate, 3.3×10^{-5} M; and glycolate, 4×10^{-2} M.

Experiment 1		Experiment 2	
Solution	Mean stomatal width, μ	Solution	Mean stomatal width, μ
Buffer control	8.3*	Buffer control	7.5
Na α-hydroxydecanesulfonate	1.1	Na α-hydroxydecanesulfonate	2.3
K glycolate + Inhibitor	2.9	Na α-hydroxydecanesulfonate + K glycolate	4.5
Glucose + Inhibitor	1.1	Na azide	1.0
KCl + Inhibitor	1.3	Na azide + K glycolate	1.0
K L-malate + Inhibitor	1.2	Phenylmercuric acetate	4.9
		Phenylmercuric acetate + K glycolate	4.8

* In the presence of each of the solutes without inhibitor, the mean stomatal widths ranged from 8.1 to 8.5 μ in this experiment.

Table II. *Effect of Anaerobic Conditions on Synthesis of Glycolic Acid by Leaf Disks in Light*

Three leaf disks were placed in each Erlenmeyer flask, and were floated on water in the light in an atmosphere of air in experiment 1 and in N_2 containing 0.03% CO_2 in experiment 2 for 60 minutes. The water was then withdrawn with a hypodermic syringe, and was replaced with a solution of 0.01 M α-hydroxy-2-pyridinemethanesulfonic acid at zero time. The experiments were carried out in the light with the disks in the atmosphere indicated and for the times shown. The glycolic acid concentration at zero time has been subtracted to show the amount that accumulated.

Expt	Atmosphere	Glycolic acid formed, μmoles/g fr wt	
		Time in inhibitor solution	
		20 min	60 min
1	Air	7.3	12.5
	N_2 + 0.03% CO_2	3.6	0.6
2	Air	4.0	9.7
	N_2 + 0.03% CO_2	0	0

taining 0.03 % CO_2 (table II). The results demonstrate the rapid and extensive accumulation of glycolic acid in the tissue when the disks were in air and a markedly diminished production of glycolic acid under comparable but anaerobic conditions. Thus an anaerobic atmosphere inhibits the synthesis of glycolic acid with concomitant diminution of the opening of stomata in the light as predicted by the hypothesis.

Effect of Concentration of CO_2 on Glycolate Synthesis and Stomatal Opening in the Light. There are several observations in the literature which indicate that glycolic acid synthesis by *Chlorella* in the light is inhibited by concentrations of CO_2 in the air in excess of 0.1 to 0.2 % (7, 17). It has also been established that at concentrations of CO_2 in excess of that normally found in air, stomatal opening is inhibited (5). It therefore became of interest to investigate the quantitative aspects of the effect of CO_2 concentration on both the synthesis of glycolic acid, in the presence of α-hydroxysulfonate, and on stomatal opening in the same tissue.

As shown in table III, 1.8 % of CO_2 in air is sufficient to inhibit greatly stomatal opening in the tobacco leaf. In experiments in which whole leaves were placed with their bases in a solution of 0.01 M α-hydroxy-2-pyridinemethanesulfonic acid in the light and the leaves were jacketed with a transparent chamber so that an atmosphere of air or 1.8% CO_2 in air could be passed over different portions of the leaf, the tissue in the atmosphere with a higher concentration of CO_2 invariably accumulated much less glycolic acid.

Similar results were obtained with leaf disks, and

Table III. *Reversal of CO_2-Induced Stomatal Closing by Glycolic Acid*

Pairs of disks in Erlenmeyer flasks were floated on water in an atmosphere of CO_2-free air in the light for 90 minutes, and were then transferred to the solutions shown for an additional 90 minutes in air containing 1.8% CO_2 in experiment 1. In experiment 2, the disks were floated on the solutions indicated for 180 minutes in the light while CO_2-free air was passed continuously through the flasks. All solutes were provided at a concentration of 0.001 M.

Solution	Experiment 1		Experiment 2	
	Mean stomatal width in each disk μ	Mean	Mean stomatal width in each disk μ	Mean
Water control*	7.3 7.2	7.3		
Water	1.3 1.5	1.4	6.6 7.7	7.2
Na glycolate, pH 4.5	3.3 3.8	3.6	6.8 7.3	7.1
Ribose-5-phosphate	1.8 2.9	2.4	6.4 7.2	6.8
Glucose	1.7 2.0	1.9	5.3 7.2	6.3
Fructose-1,6-diphosphate	1.3 2.5	1.9	6.4 7.8	7.1
Sucrose	1.3 1.8	1.6	7.1 7.1	7.1
L-Serine	1.5 1.5	1.5	7.3 7.8	7.6
NaCl	1.0 1.4	1.2	5.8 7.3	6.6

* These disks were floated on water in CO_2-free air for the entire 180 min.

one such experiment is shown in figure 3. There was little difference between air or 0.074 % CO_2 in air on either stomatal opening or the extent of accumulation of glycolic acid, but at a concentration of 0.50 % CO_2 in air there was a large inhibition of stomatal opening and an equal effect on glycolic acid synthesis. Thus it appears that the concentrations of CO_2 in air that inhibit stomatal opening are very similar to those that bring about a parallel inhibition of the synthesis of glycolic acid.

Reversal of CO_2-Induced Closing by Glycolic Acid. If the closing of stomata at higher partial pressures of CO_2 in the light is the result of an inhibition of the synthesis of glycolic acid, then it seemed reasonable to expect that supplying glycolic acid to the leaf disks might overcome this deficiency and reverse the stomatal closing. Numerous experiments of this kind were performed, and glycolate invariably acted in the predicted manner. It stim-

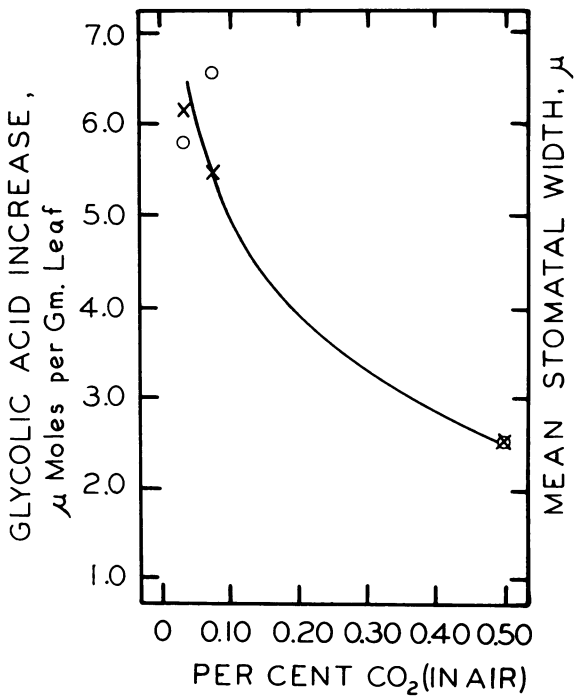

FIG. 3. Effect of CO_2 concentration in air on synthesis of glycolic acid and on stomatal aperture. Tobacco leaf disks were floated on water (for determination of stomatal aperture) or on 0.01 M α-hydroxy-2-pyridine-methanesulfonic acid (6 disks for each determination of glycolic acid) for 120 minutes in the light in air, 0.074 % CO_2 in air, or 0.50 % CO_2 in air. ○, mean stomatal width; ×, glycolic acid accumulated compared with the zero time sample.

ulated stomatal opening in the presence of concentrations of CO_2 higher than normal. Sometimes, however, glucose and even certain inorganic salts would reverse the closing brought about by CO_2. In order to demonstrate the reversal by glycolate with a high order of specificity, it was necessary to lower the concentration of solute on which the disks floated to about 0.001 M, and to place the disks with the solution to be tested in CO_2-free air for a preliminary period in the light. When these precautions were taken, glycolic acid was clearly found to be superior to a number of other metabolites in reversing the CO_2-induced stomatal closure, whereas the other metabolites had little or no effect on stomatal opening in CO_2-free air (table III). This experiment also illustrates the variability of stomatal apertures that can be expected in each of the pair of disks in a given treatment.

The results demonstrate the superiority of glycolic acid as a substrate in reversing CO_2-induced closure of stomata in the light, and thus further support the hypothesis that high concentrations of CO_2 close stomata because the synthesis of glycolic acid is interfered with under these conditions.

Discussion

Stomata in tobacco leaf disks open normally to their maximal extent in the light in the presence of O_2 and the low concentrations of CO_2 normally present in the atmosphere. We have now demonstrated that these conditions are also essential for the maximal rate of synthesis of glycolic acid. The reversal of the CO_2-induced closing by glycolic acid also suggests that at higher concentrations of CO_2 the synthesis of glycolic acid is limited and stomatal opening is thereby diminished. Highly specific inhibitors such as the α-hydroxysulfonates interfere with the further metabolism of glycolic acid and this also prevents stomatal opening. The experiments described support the view that these inhibitors act on stomata because of their action on the glycolate oxidase enzyme in the tissue.

We assume that stomatal opening in the light is an active process by which water is pumped into the guard cells to increase their turgor. The striking temperature dependence of stomatal opening (12, 15) when the problem of water deficits is eliminated, and the lack of an apparent temperature effect on the rate of stomatal closing in the dark (24) also support this view.

In the classical explanation of stomatal opening, it was assumed that the carbohydrate concentration in the guard cells increases in the light and an osmotic pump is thus established. Although this hypothesis has not received strong experimental support, it is possible that glycolic acid plays a special role in the synthesis of carbohydrate in the guard cells. It is now well established that glycolic acid can serve as a precursor of carbohydrate in leaves (6, 16).

Glycolic acid may also be involved in a pump that utilizes ATP, or a compound in equilibrium with this substance, produced by photosynthetic phosphorylation in the chloroplasts. For example, in experiments on the anaerobic uptake of glucose by *Chlorella* in the light, Butt and Peel (3) obtained evidence that the glycolate-glyoxylate cycle participated in the generation of ATP that was essential for the uptake of glucose by the cells in the following sequence of reactions:

$$\underset{\text{glyoxylate}}{CH_2OH\text{-}COO^-} + 0.5\ O_2 \xrightarrow[\alpha\text{-hydroxysulfonates}]{} \underset{\text{glycolate}}{CHO\text{-}COO^-} + H_2O \qquad \text{I}$$

$$CHO\text{-}COO^- + NADPH_2 \longrightarrow CH_2OH\text{-}COO^- + NADP \qquad \text{II}$$

$$NADP + ADP + P_i + H_2O \xrightarrow{\text{light}} NADPH_2 + ATP + 0.5\ O_2 \qquad \text{III}$$

Reaction I represents the glycolate oxidase reaction in the presence of excess catalase. It is inhibited competitively by α-hydroxysulfonates. Reaction II has recently been demonstrated by Zelitch and Gotto (26), who showed that a reduced nicotinamide-adenine dinucleotide phosphate-linked glyoxylate reductase is present in tobacco and spinach leaves, and the enzyme has properties considerably different from the $NADH_2$-glyoxylate reductase previously studied in the same tissues. Reaction III represents

the noncyclic photophosphorylation described by Arnon et al. (1). These reactions demonstrate the manner in which the synthesis and metabolism of glycolic acid may be essential for the reoxidation of $NADPH_2$, a process which is necessary in order for reaction III to proceed with its resulting ATP synthesis. The glycolate-glyoxylate cycle would be especially important at low concentrations of CO_2, when less of the $NADPH_2$ may be oxidized in the course of CO_2 reduction. Although glycolic acid concentration was determined in the entire tissue, we assume that the pump shown in reactions I to III would operate in the guard cells.

Surprisingly, although the importance of glycolic acid in plant metabolism is becoming increasingly evident, the mechanism by which it is synthesized in photosynthetic tissues is still uncertain. For example, it has been suggested that it is derived from a nonphosphorylated 2-carbon fragment originating in the photosynthetic cycle (18), from a phosphorylated 2-carbon fragment (8), and by an undefined but presumably novel carboxylation reaction (17). Considerably more experimental work will be needed to establish the steps in its synthesis.

Summary

Inhibitors of the oxidation of glycolic acid, α-hydroxysulfonates, inhibited stomatal opening in the light in tobacco leaf disks at concentrations similar to those which cause glycolic acid to accumulate in the same tissue. Stomatal closing by α-hydroxysulfonates was reversed in a competitive manner by glycolic acid supplied to the leaf disks, as had previously been shown to occur with the isolated glycolate oxidase.

O_2 in the atmosphere surrounding the tissue is essential for rapid rates of stomatal opening in the light and also for the synthesis of glycolic acid. At greater than normal concentrations of CO_2 in air, stomata close in the light and glycolic acid synthesis is also inhibited. The CO_2-induced closing of stomata was reversed by providing glycolic acid to the leaf disks.

It was concluded that the results are consistent with a requirement for the synthesis and oxidative metabolism of glycolic acid in the process of stomatal opening in the light.

Acknowledgments

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Control by Iron of Chlorophyll Formation and Growth in Euglena gracilis [1, 2]

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Iron has long been recognized as an essential element for growth and chlorophyll formation in higher plants (2) and, more recently, for photosynthetic bacteria (7, 8, 9).

Despite a wide variety of studies on iron deficiency chlorosis (1, 18), there is no specific information on the site of action of iron in chlorophyll formation. The purpose of our study was to identify this site or sites.

The use of higher plants for physiological and biochemical studies presents certain disadvantages. For reasons outlined elsewhere (15), the alga *Euglena gracilis* combines certain favorable characteristics of higher plants and microorganisms for investigations involving trace metals. Since, in addition, the physiology of chlorophyll production in *Euglena* at least superficially resembles that in higher plants (20) and an iron requirement for growth has been established (13), we thought that *Euglena* might be especially suitable for investigating the role of iron in chlorophyll formation.

In the present paper we report on some of the physiological variables concerned in the control of chlorophyll synthesis and growth by iron. We shall show that the iron-dependent chlorophyll-forming system of *Euglena* provides a remarkable test system for the study of the means by which an inorganic ion controls a metabolic process.

Methods and Materials

Euglena gracilis (Klebs), *Z* strain (Hutner), was originally obtained from Dr. S. Hutner.

Growth Conditions. The culture medium was as described previously (4, 15). The culture flasks were shaken at 25° in a Model G-25 Rotary Shaker (New Brunswick Scientific Company), modified so that the cultures could be illuminated through a large window in the top of the shaker. Two 22-w daylight fluorescent lamps were suspended to provide 25 or 50 ft-c illumination at the level of the culture media. Growth was monitored turbidimetrically in corrected Klett units (K_c) (15).

All subsequent incubations were carried out at 25°.

Purification of Medium Components, Glassware, etc. Potassium phosphate, magnesium sulfate, calcium nitrate, malic acid, and the surfaces of culture flasks and other containers were purified as described elsewhere (15). Deionized water, hydrochloric acid, and ammonium hydroxide were prepared according to Thiers (19). Glutamic acid was purified as follows: reagent grade glutamic acid was made M in metal-free hydrochloric acid and 1.5 M

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