Plant Physiol. (1974) 53, 395-397

A Study of the Control of Glycolate Excretion in *Chlorella*[1]

Received for publication August 22, 1973 and in revised form November 7, 1973

BRIAN COLMAN, A. G. MILLER, AND B. GRODZINSKI
Department of Biology, York University, Downsview, Ontario M3J 1P3, Canada

ABSTRACT

***Chlorella pyrenoidosa* cells grown on 5% CO_2 excreted glycolate when incubated in light with 10 mM bicarbonate, but excreted no glycolate under the same conditions when they were maintained on air for 7 hours prior to the assay. Incubation of 5% CO_2-grown and air-grown cells with 10 mM isonicotinyl hydrazide or 10 mM α-hydroxypyridinemethane sulfonate during the assay stimulated the excretion of glycolate by CO_2-grown cells severalfold that of air-grown cells.**

Adaptation of CO_2-grown *Chlorella* to growth on air did not affect the levels of glycolate dehydrogenase in the cells and did not affect the rate of dark oxidation and metabolism of exogeneous ^{14}C-glycolate by the cells. These results indicate that the lack of glycolate excretion by air-grown or air-adapted cells of *Chlorella* cannot be explained by a concomitant change in the level of glycolate dehydrogenase.

Under certain conditions, a number of green algae excrete a large proportion of photosynthetically fixed CO_2 as glycolate (10, 14, 17–19, 20). In all cases where glycolate excretion has been found, the cells have been grown on high levels of CO_2 (0.2-5%) followed by measurement of glycolate excretion at lower CO_2 levels and higher light intensities. For instance, Watt and Fogg (21) showed that *Chlorella pyrenoidosa* cells grown on air rather than CO_2-enriched air failed to excrete glycolate under assay conditions where high CO_2-grown cells excreted large amounts.

Nelson and Tolbert (15) were able to convert *Chlamydomonas reinhardtii* cells, grown on 1% CO_2 in air and having a high rate of glycolate excretion under assay conditions of 6.5 mM bicarbonate in the light, to cells incapable of excreting glycolate under these same conditions. This conversion was accomplished by bubbling the 1% CO_2-grown cells with air for 20 hr preceding the measurement of the excretion rate. During the aeration period, they reported an increase in the level of glycolate-DCIP[2] oxidoreductase activity concomitant with the decrease in the glycolate excretion rate and suggested that these two events were causally related. Similarly, Codd *et al.* (4) have shown that *Chlamydomonas, Euglena,* and *Chlorella,* grown on 5% CO_2, have lower levels of glycolate-DCIP oxidoreductase than the same algae grown on air alone.

However, Cooksey (5) has demonstrated that the level of the enzyme in *Chlamydomonas* is not regulated solely by CO_2 tension but is partly dependent on the availability of nitrogen to the cell. Further it has been shown in *Euglena* that while glycolate excretion occurs at all stages of the cell cycle there is sufficient glycolate dehydrogenase present to oxidize all the glycolate formed (3). The present study provides evidence that more glycolate is formed and metabolized in *Chlorella* maintained on high CO_2 tension than cells in air and further shows that no detectable changes in the levels of the enzyme occur in cells which lose their ability to excrete glycolate.

MATERIALS AND METHODS

Chlorella pyrenoidosa Chick was obtained bacteria-free from the University of Indiana Culture Collection, Bloomington, Indiana. Cultures for experimental use were grown axenically as previously described (13) on the medium of Watt and Fogg (21) modified as follows. KNO_3 (0.5 gm/l) replaced urea as the nitrogen source; 1% ammonium ferric citrate (0.5 ml/l) was the iron source, and 1.0 ml of Gaffron's minor elements (11) was the source of micronutrient. Cultures were bubbled with air or 5% CO_2 in air.

For the measurement of the rate of glycolate excretion, cells were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 8.0), then resuspended in 2.4 ml of the buffer, 0.3 ml of 0.1 M $NaHCO_3$, and 0.3 ml of distilled water or 0.1 M INH (K & K Laboratories Inc., Plainview, N.Y.) or 0.1 M HPMS (Aldrich Chemical Co., Milwaukee, Wis.) contained in 25-ml Erlenmeyer flasks. The flasks, each containing 10 to 20 mg dry weight of cells, were then incubated for 60 min at 28 C and illuminated from the bottom with photoflood lamps giving a light intensity of 12 klux at flask level. After incubation the cells were recovered from the medium by centrifugation at 500*g* for 5 min followed by further centrifugation of the supernatant at 10,000*g* for 15 min. Glycolate in the medium was estimated using the method of Calkins (1).

Uptake and oxidation of ^{14}C-glycolate by intact cells was determined by the methods of Miller *et al.* (13). The activity of glycolate-DCIP oxidoreductase was measured in crude homogenates in the presence of DCIP by determination of glyoxylate formation as described by Grodzinski and Colman (8). The crude enzyme preparation was made by the method of Nelson and Tolbert (16).

RESULTS AND DISCUSSION

Glycolate Excretion. *Chlorella* cells grown on 5% CO_2 in air excreted large amounts of glycolate into the surrounding medium when harvested from the growth culture and incubated in light with bicarbonate (Fig. 1). However, 5% CO_2-grown *Chlorella* lost this characteristic over 4 to 7 hr when the growth culture was put on air-bubbling (Fig. 2). This finding agrees with the results of Nelson and Tolbert (15). Cells grown on

[1] This work was supported by grants from the National Research Council of Canada.

[2] Abbreviations: DCIP: 2,6-dichlorophenolindophenol; INH: isonicotinyl hydrazide; HPMS: α-hydroxypyridinemethane sulfonate.

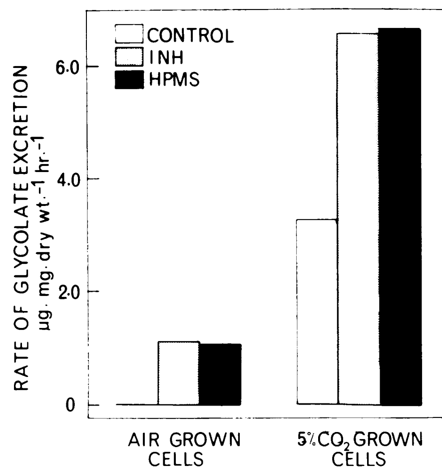

FIG. 1. Effect of 10 mM INH and 10 mM HPMS on the rate of glycolate excretion by *Chlorella* grown on 5% CO_2 or on air.

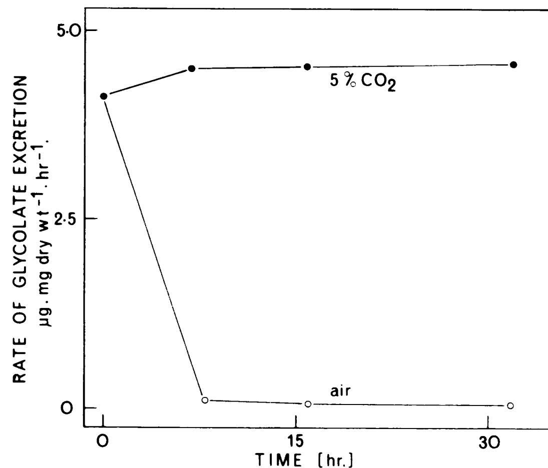

FIG. 2. Time course of the change in glycolate excretion rate of cells of a *Chlorella* culture, grown on 5% CO_2, after half of the culture was transferred to air (○) while the other half was maintained on 5% CO_2 (●).

Table I. *Effect of Isonicotinyl Hydrazide on the Rate of Glycolate Excretion by Air-grown Chlorella during Adaptation to 5% CO_2 in air*

Time on 5% CO_2	Glycolate excreted	
	Control	INH (10 mM)
hr	*µg/mg dry wt·hr*	
4	0.05	2.5
14	0.05	2.5
33	1.1	6.8
70	3.8	17.3

air rather than 5% CO_2 in air did not excrete glycolate when harvested and resuspended in 10 mM bicarbonate in the light (Fig. 1). Cells grown on air could be converted to cells having a high rate of glycolate excretion by bubbling the growth culture with 5% CO_2 in air, but the conversion took considerably longer than the 5% CO_2 to air conversion (Table I).

The presence of INH during the assay stimulated the excretion of glycolate by both 5% CO_2-grown and air-grown cells (Fig. 1). It has been well established that INH greatly stimulates the rate of glycolate excretion by *Chlorella* cells grown on 4% CO_2 in air (17). Nelson and Tolbert (15) have shown that INH also stimulates glycolate excretion by air-grown *Chlamydomonas* and have used this observation to suggest that lack of glycolate excretion by air-grown cells is not due to lack of formation of glycolate, but no comparison was made with the effect of INH on 5% CO_2-grown cells. The increase in glycolate excretion in 5% CO_2-grown *Chorella* caused by INH was considerably greater than that in air-grown cells (Fig. 1.) Similarly, HPMS caused an increase in excretion by 5% CO_2-grown *Chlorella* greater than that in air-grown cells presumably due to the inhibition of further metabolism of glycolate (Fig. 1). HPMS is an inhibitor of glycolate-oxidizing enzymes of both higher plants (23) and algae (8) and has been shown to stimulate glycolate excretion in algae (2, 3, 6, 12). These results support the finding that air-grown cells produce glycolate

Table II. *Activity of glycolate-DCIP oxidoreductase in cells of Chlorella*

Enzymic activity was measured in extracts of cells grown on 5% CO_2 and in cells of the same culture 12 hr after half of the culture had been transferred to air and the other half maintained on 5% CO_2. Enzyme activities were estimated by determining glyoxylate phenylhydrazone formation at 324 nM after incubating the cell extract (approximately 0.25 mg of protein) with 60 µmoles potassium glycolate, 0.2 µmole DCIP, in 0.1 M potassium phosphate buffer, pH 8.6, to a final volume of 3.0 ml at 28 C for 15 min.

Time after Transfer	Glycolate-DCIP oxidoreductase Activity			
	5% CO_2	Air	5% CO_2	Air
hr	*µmoles glyoxylate formed min⁻¹/mg protein*		*µmoles glyoxylate formed min⁻¹/10⁹ cells*	
0	3.95	—	9.90	
12	3.47	3.67	8.99	8.63

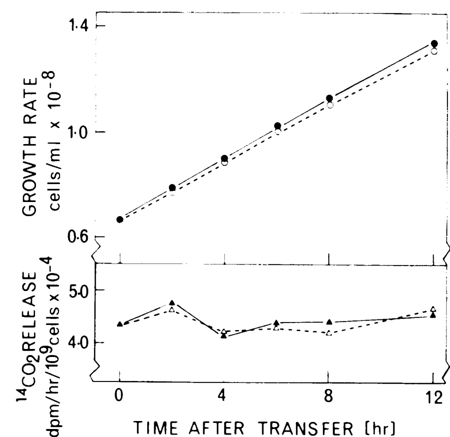

FIG. 3. Rate of dark oxidation of ^{14}C-glycolate to $^{14}CO_2$ by aliquots (1.35×10^9 cells) of a 5% CO_2-grown *Chlorella* culture taken at various times after transfer of one half of the culture to air (△---△) while maintaining the other on 5% CO_2 (▲——▲) as a control. Growth rates of that portion of the culture transfered to air (○---○) and that maintained on 5% CO_2 (●——●) are shown above. The washed cells were incubated in 4.0 ml of 20 mM potassium phosphate buffer, pH 5.5, containing 1.8 µc glycolic acid-1-^{14}C (0.19 µmole), and 100 µmoles of potassium glycolate for 1 hr at 28 C in the dark. $^{14}CO_2$ released was trapped in 20% KOH and assayed as described previously (13).

while not excreting it (15), but also show that 5% CO_2-grown cells produce considerably more glycolate than is excreted and in fact do metabolize more glycolate than cells grown only in air. This is inconsistent with the concept that the activity of an enzyme of further glycolate metabolism, *e.g.* glycolate-DCIP oxidoreductase limits the utilization of glycolate in the cell and is the cause of massive glycolate excretion (15).

Glycolate oxidation. The activity of glycolate-DCIP oxidoreductase was assayed in cells grown on 5% CO_2, the culture was divided, and while one-half was maintained on 5% CO_2, the other half was transferred to air. After 12 hr under these conditions, the activity of the enzyme in the cells of the two cultures was again determined (Table II). These data indicate that even after 12 hr on air, when massive glycolate excretion can no longer be observed (Fig. 2), there are no marked differences between the level of the glycolate-oxidizing enzyme in air-grown and 5% CO_2-grown cells. In all these assays the initial rate of reaction was proportional to protein concentration. Similar to other studies (3–5, 15), these determinations necessitated the use of an artificial electron acceptor, a procedure which may not accurately reflect the *in vivo* activity of this enzyme. Although the natural hydrogen acceptor for this reaction is not known (4, 9, 16), the use of artificial electron acceptors indicates little difference in the activity of this enzyme in response to CO_2 concentration.

In order to obtain some measure of the *in vivo* activity of this enzyme, the ability of whole cells to oxidize exogenous glycolate to CO_2 in the dark was examined. Similar to blue-green algae (13), *Chlorella* oxidized most of the supplied ^{14}C-glycolate to $^{14}CO_2$ in the dark and this oxidation was inhibited by the addition of 10 mM HPMS to the cells. The rate of glycolate oxidation by cells of a culture grown on 5% CO_2 was determined and then subsequently determined periodically for a further 12 hr after half of the culture had been transferred to air. At low concentrations of carrier glycolate, over 90% of the ^{14}C-glycolate taken up by the cells was oxidized to $^{14}CO_2$ in the dark. In these experiments a high concentration of carrier was used so that the rate of oxidation not the rate of uptake by the cells would be the limiting rate and under these conditions only 50 to 60% of the ^{14}C-glycolate taken up by the cells was oxidized to $^{14}CO_2$. Over a period of 12 hr after the transfer of cells to air, the rate of glycolate oxidation did not change markedly and did not differ significantly from that of cells maintained on 5% CO_2 (Fig. 3). In addition, the rate of uptake and incorporation of radioactivity from ^{14}C-glycolate by cells remained relatively constant over this same 12-hr period, both in cells maintained on 5% CO_2 and those adapted to air. The rate of growth of the culture during the course of this experiment was similar to that normally observed in log phase cultures of this alga. Such a constant growth rate over this period indicates that nutrient supply was adequate and that the inability to observe a change in enzyme levels (Table II) or in rates of glycolate oxidation (Fig. 3) is not due to nitrogen limitation (5).

The data indicate that the oxidation of glycolate is not affected by changes in the concentration of CO_2 supplied to the algae, and that in cells grown either on high or low CO_2 more glycolate is being metabolized by the cells than is being excreted. Since algal cells when maintained at the same CO_2 concentration under which they were grown excrete little glycolate (17, 18, 21), excretion appears to be a temporary phenomenon occurring after a transfer of cells from a high to low CO_2 concentration. Excretion may therefore result from a concomitant change in the rate-limiting step of the reductive pentose cycle causing an increase in the rate of glycolate synthesis due to the breakdown of a glycolate precursor (22). Cessation of excretion would then occur when the pool of glycolate precursor had been depleted. Alternatively, excretion may be due to a lack of available CO_2 for photosynthesis, since it has been demonstrated that carbonic anhydrase formation is suppressed in *Chlorella* and *Chlamydomonas* grown on 5% CO_2 and after transfer of these cells to 0.03% CO_2 photosynthesis is inhibited for 90 min (7). These conditions would favor the formation of excessive amounts of glycolate and the induction of carbonic anhydrase would cause a subsequent decrease of glycolate formation and the cessation of excretion.

While changes in levels of glycolate-DCIP oxidoreductase occur in response to changing CO_2 levels (4, 15), these changes, especially those reported for *Chlorella,* are not of a sufficient magnitude to account for the cessation of glycolate excretion. The control of glycolate excretion in *Chlorella* may be due to CO_2 concentration, not by a direct effect on glycolate-DCIP oxidoreductase, but by its effect on enzymes which directly or indirectly control the synthesis of glycolate.

Acknowledgment—We wish to thank R. K. Ingle for able technical assistance.

LITERATURE CITED

1. Calkins, V. P. 1943. Microdetermination of glycolic and oxalic acids. Ind. Chem. Anal. Ed. 15: 762–763.
2. Cheng, K. H., A. G. Miller, and B. Colman. 1972. An investigation of glycolate excretion in two species of blue-green algae. Planta 103: 110–116.
3. Codd, G. A. and M. J. Merrett. 1971. The regulation of glycolate metabolism in division synchronized cultures of *Euglena*. Plant Physiol. 47: 640–643.
4. Codd, G. A., J. M. Lord, and M. J. Merrett. 1969. The glycolate oxidizing enzyme of algae. FEBS Lett. 5: 341–342.
5. Cooksey, E. 1971. Glycolate-dichlorophenolindophenol oxidoreductase in *Chlamydomonas reinhardtii*. Plant Physiol. 48: 267–269.
6. Gimmler, H., W. Ullrich, J. Domanski-Kaden, and W. Urbach. 1969. Excretion of glycolate during synchronous culture of *Ankistrodesmus braunii* in the presence of disalicylidenepropanediamine or hydroxypyridinemethane sulfonate. Plant Cell Physiol. 10: 103–112.
7. Graham, D., C. A. Atkins, M. L. Reed, B. D. Patterson, and R. M. Smillie. 1971. Carbonic anhydrase, photosynthesis and light induced pH change. *In:* M. D. Hatch, C. B. Osmond and R. O. Slatyer, eds., Photosynthesis and Photorespiration. Wiley Interscience, New York, pp. 267–274.
8. Grodzinski, B. and B. Colman. 1970. Glycolic acid oxidase activity in cell-free preparations of blue-green algae. Plant Physiol. 45: 735–737.
9. Grodzinski, B. and B. Colman. 1972. Disc electrophoresis of glycolate-oxidizing enzymes. Phytochemistry 11: 1281–1285.
10. Hess, J. L., N. E. Tolbert, and L. M. Pike. 1967. Glycolate biosynthesis by *Scenedesmus* and *Chlorella* in the presence or absence of $NaHCO_3$. Planta 74: 278–285.
11. Hughes, E. O., P. R. Gorham, and A. Zehnder. 1958. Toxicity of a unialgal culture of *Microcystis aeruginosa*. Can. J. Microbiol. 4: 225–236.
12. Lord, J. M., G. A. Codd, and M. J. Merrett. 1970. The effect of light quality on glycolate formation and excretion in algae. Plant Physiol. 46: 855–856.
13. Miller, A. G., K. H. Cheng, and B. Colman. 1971. The uptake and oxidation of glycolic acid by blue-green algae. J. Phycol. 7: 97–100.
14. Miller, R. M., C. M. Meyer, and H. A. Tanner. 1963. Glycolate excretion and uptake by *Chlorella*. Plant Physiol. 38: 184–188.
15. Nelson, E. B. and N. E. Tolbert. 1969. The regulation of glycolate metabolism in *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta 184: 263–270.
16. Nelson, E. B. and N. E. Tolbert. 1970. Glycolate dehydrogenase in green algae. Arch. Biochem. Biophys. 141: 102–110.
17. Pritchard, G. G., W. J. Griffin, and C. P. Whittingham. 1962. The effect of carbon dioxide concentration, light intensity and isonicotinyl hydrazide on the photosynthetic production of glycolic acid by *Chlorella*. J. Exp. Bot. 13: 176–184.
18. Stabenau, H. 1972. Über die ausscheidung von Glykolsäure bei *Chlorogonium elongatum* Dangeard. Biochem. Physiol. Pflanzen 163: 42–51.
19. Tolbert, N. E. and L. P. Zill. 1956. Excretion of glycolic acid by algae during photosynthesis. J. Biol. Chem. 222: 895–906.
20. Warburg, O. and G. Krippahl. 1960. Glykolsäurebildung in *Chlorella*. Z. Naturforschung. 15b: 197–199.
21. Watt, W. D. and G. E. Fogg. 1966. The kinetics of extracellular glycolate production by *Chlorella pyrenoidosa*. J. Exp. Bot. 17: 117–134.
22. Wilson, A. T. and M. Calvin. 1955. The photosynthetic cycle: CO_2 dependent transients. J. Amer. Chem. Soc. 77: 5948–5957.
23. Zelitch, I. 1957. α-Hydroxysulfonates as inhibitors of enzymatic oxidation of glycolic and lactic acids. J. Biol. Chem. 224: 251–260.