Plant Physiol. (1968), 43, 289–291

Short Communication

Glycolate Oxidase Activity in Algae

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Received October 11, 1967.

The glycolate oxidase reaction by which glycolate is oxidized to glyoxylate (3, 10) is catalyzed by a flavoprotein (18) that is widely distributed in higher plants (12) and fungi (4). Its presence is generally assumed in all photosynthetic tissues, since glycolate is an early product of photosynthesis (14), yet this substrate is usually found in only minute amounts. The oxidation of glycolate in higher plants has been shown to be part of a pathway leading to the synthesis of carbohydrate (9, 13); it may be concerned with non-cyclic photophosphorylation (1); the reaction plays a role in the opening of leaf stomata (19); and it is important in photorespiration (7, 17). It was therefore surprising that Hess and Tolbert (8) recently failed to detect any glycolate oxidase activity in several algae including *Chlamydomonas* and *Chlorella*. It is not certain whether the method of culture or the extraction procedure or their assay was at fault, since we have readily found glycolate oxidase activity in both species of algae. In *Chlamydomonas* the activity is in fact almost the same as in homogenates of tobacco leaves when calculated on a chlorophyll basis.

Culture of Chlamydomonas and Chlorella. Our cultures of *Chlamydomonas reinhardi*, strain 137c mt^+, were kindly provided by Dr. N. W. Gillham, Harvard University, and the *Chlorella pyrenoidosa* Tx 71105 by Dr. J. Myers, University of Texas, Austin. Cultures were grown in Levine and Ebersold's liquid minimal medium (11) in 600 ml volumes contained in 1 liter Erlenmeyer flasks at 25°. Each flask was equipped with a sintered glass sparger through which filtered air was passed. The cultures were illuminated continuously with fluorescent light at an intensity of about 1500 ft-c. The inoculum consisted of a cell suspension prepared from an agar culture grown on yeast extract-acetate agar. All cultures were harvested after 5 days, and routinely checked for absence of bacterial contamination with a phase contrast microscope.

Preparations of Glycolate Oxidase. About 600 ml of a suspension of *Chlamydomonas* cells containing 2.4 to 3.5 $\times$ 10^6 cells per ml were centrifuged for 5 minutes at 2000 $\times$ g at 15°. The cells were suspended in 6 ml of 0.01 M potassium phosphate buffer (pH 7.0) and the final volume was about 10 ml. The suspension was frozen overnight at −15°. The frozen mass was thawed, placed on ice and disintegrated sonically for 45 seconds at maximum power with a Biosonik II probe. Examination under the microscope revealed mainly particles about 1 μ in size and few whole cells. This suspension was centrifuged at 10,000 $\times$ g at 5° for 4 minutes, and the dark green residue was discarded. The turbid green supernatant fluid was again frozen overnight at −15°. After thawing, the homogenate was centrifuged for 10 minutes at 10,000 $\times$ g at 5°, yielding a clear yellow supernatant fraction (7 ml) and a green residue fraction. The green residue fraction was suspended in 7 ml of cold water and gently homogenized in a Ten Broeck homogenizer.

Chlorella cells were treated in a similar manner. Sonic disintegration was carried out for 8 minutes after the addition of alumina. Even then not many of the cells were disrupted by the treatment. The homogenate was decanted from the alumina and was frozen, and a supernatant and a residue fraction were obtained.

Tobacco plants (*Nicotiana tabacum*, var. Havana seed) were grown in a subirrigated bench in the greenhouse. A homogenate active in glycolate oxidase was prepared by grinding 0.5 g fresh weight of leaf in 4.5 ml of 0.1 M triethanolamine hydrochloride (pH 8.0) in a Ten Broeck homogenizer in the cold (16).

Glycolate Oxidase Assay. A sensitive assay was used based upon the well known ability of glycolate oxidase to utilize 2,6-dichlorophenolindophenol as a hydrogen acceptor (6, 18). The reaction was carried out in Thunberg tubes, and the rate of dye reduction was measured at 590 mμ in a Coleman colorimeter. The contents of the Thunberg tubes in a typical assay are given in the legend to figure 1. One unit of activity was taken as the amount of enzyme causing a decrease in absorbancy of 0.01 per minute at 25°. This is equivalent to about 0.002 μmole of glycolate oxidized. The change in absorbancy between 3 and 13 minutes after tipping in the enzyme was used to determine the reaction rate, and a correction was always made

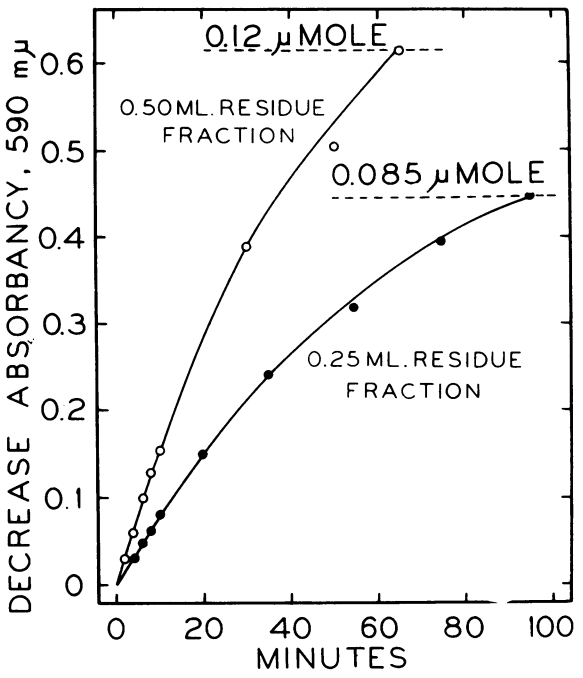

FIG. 1. Effect of concentration of residue fraction from *Chlamydomonas* on rate of glycolate oxidation and stoichiometry of the reaction. The Thunberg tubes contained 200 μmoles of potassium phosphate buffer at pH 7.0, 60 μmoles potassium glycolate, 0.16 μmole 2,6-dichlorophenolindophenol and water to make the final volume 6.0 ml; 0.25 ml or 0.50 ml of the green residue fraction as indicated was placed in the side arm. The tube was evacuated with a water pump for 1 minute, and after several minutes the enzyme was tipped in to start the reaction. A tube without glycolate served as control in each experiment. The change in absorbancy was measured in a Coleman colorimeter at 590 mμ at approximately 25°. The glyoxylate produced in each experiment is given in the text.

for any activity occurring in the absence of added glycolate. Protein was estimated from Kjeldahl nitrogen determinations (for which we are grateful to Dr. S. Patil), and chlorophyll determinations were made on acetone extracts at 652 mμ (2).

Comparison of Glycolate Oxidase Activity in Supernatant and Residue Fractions. Table I shows that glycolate oxidase activity was present in *Chlamydomonas* and *Chlorella*. When compared at pH 7.0, on a chlorophyll basis there is as much activity in *Chlamydomonas* as in tobacco leaf, while *Chlorella* has less activity when calculated on a protein basis. However, at pH 8.0 the activity from tobacco leaf increased 2.7-fold, while the activity in *Chlamydomonas* and *Chlorella* was unchanged. In tobacco leaves, the glycolate oxidase is readily extracted (3, 16), although in both species of algae examined most of the glycolate oxidase activity remained in the green residue fraction. Addition of riboflavin phosphate (0.05 to 0.10 mg), which is known to be the prosthetic group of glycolate oxidase in leaves, slightly decreased the activity of the glycolate oxidase from the algae.

Stoichiometry of the Glycolate Oxidase Reaction in Chlamydomonas. Figure 1 shows that the initial rate of reduction of 2,6-dichlorophenolindophenol in the presence of glycolate is proportional to the amount of the green residue fraction added. At the end of the experiments pictured in figure 1, the glyoxylate formed was determined by a modification of the method of Friedemann and Haugen (5), which is based on formation of the 2,4-dinitrophenylhydrazone. In the absence of glycolate, 0.001 μmole of dye was reduced in each experiment. Where 0.12 μmole of dye was reduced, 0.14 μmole of glyoxylate was formed; in the experiment in which 0.085 μmole of dye was reduced, 0.092 μmole of glyoxylate appeared. The results thus show

Table I. *Glycolate Oxidase Activity of Fractions from Algae and Tobacco Leaf Homogenate*

The assays were carried out in Thunberg tubes as described in the text and in figure 1. A comparison of the activity at pH 8.0 compared with that at pH 7.0 for the green residue fraction of the algae and the tobacco homogenate is given in the last column. A unit of enzyme activity is the amount causing a decrease in absorbancy of 0.01 per minute between 3 and 13 minutes at 25°.

	Homogenate	Clear supernatant	Green residue	Ratio $\frac{\text{pH 8.0}}{\text{pH 7.0}}$
Chlamydomonas, 1.44 × 10^9 cells				
Total units	26	3.0	23	1.0
Units per mg protein			4.4	
Units per mg chlorophyll	28			
Chlorella, 1.08 × 10^{10} cells				
Total units	53	16	38	0.86
Units per mg protein		1.3	2.6	
Tobacco leaf, 0.5 g fr wt				
Total units	41			2.7
Units per mg chlorophyll	29			

that for each mole of dye that is reduced in the presence of glycolate, approximately 1 mole of glyoxylate is synthesized.

Synthesis of Glyoxylate-^{14}C from Glycolate-^{14}C by Residue Fraction from Chlamydomonas. As further evidence that glyoxylate is the product of this glycolate oxidase reaction, experiments were carried out similar to those in figure 1 in which the substrate consisted of 30 μmoles of potassium glycolate-2-^{14}C purified on Dowex-1 acetate (15) and containing 2.16×10^6 CPM. The enzyme was 0.4 unit of the residue fraction or the residue fraction after being heated on a steambath for 5 minutes. After 105 minutes, 4 ml of 95% ethyl alcohol was added to the Thunberg tubes. The suspension was centrifuged and the glyoxylic acid was separated on a column of Dowex-1 acetate (15). The radioactivity was determined by scintillation counting. The Thunberg tube containing boiled enzyme showed 0.003 μmole of dye reduced and was treated in the same way and served as control. In the experimental tube the dye reduced equalled 0.041 μmole and 0.035 μmole of glyoxylate-^{14}C was formed, in reasonable agreement with the expected stoichiometry.

Acknowledgments

The skillful technical assistance of Bonnie Gambs and Sandra Ringo is gratefully acknowledged.

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