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Synthesis, Excretion, and Metabolism of Glycolate under Highly Photorespiratory Conditions in *Euglena gracilis* Z[1]

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ABSTRACT

Glycolate was excreted from the 5% CO_2-grown cells of *Euglena gracilis* Z when placed in an atmosphere of 100% O_2 under illumination at 20,000 lux. The amount of excreted glycolate reached 30% of the dry weight of the cells during incubation for 12 hours. The content of paramylon, the reserve polysaccharide of *E. gracilis*, was decreased during the glycolate excretion, and of the depleted paramylon carbon, two-thirds was excreted to the outside of cells and the remaining metabolized to other compounds, both as glycolate. The paramylon carbon entered Calvin cycle probably as triose phosphate or 3-phosphoglycerate, but not as CO_2 after the complete oxidation through the tricarboxylic acid cycle. The glycolate pathway was partially operative and the activity of the pathway was much less than the rate of the synthesis of glycolate in the cells under 100% O_2 and 20,000 lux; this led the cells to excrete glycolate outside the cells. Exogenous glycolate was metabolized only to CO_2 but not to glycine and serine. The physiologic role of the glycolate metabolism and excretion under such conditions is discussed.

Glycolate is an early product of photosynthesis in photosynthetic organisms (4). Production of glycolate increases when the CO_2 concentration is lowered and the O_2 concentration is increased (4). In higher plants, glycolate is metabolized to form carbohydrate through the glycolate pathway (15, 25); the metabolism of glycolate is referred to as photorespiration because of the uptake of O_2 and the evolution of CO_2 in the pathway (25).

It has been proposed that a major physiologic role of photorespiration in higher plants is the dissipation of excess light energy captured during photosynthesis in low CO_2 and high O_2 concentrations and at high light intensity through the metabolism of glycolate (25). This dissipation of excess energy also may occur through elevation of the cellular concentration of CO_2 by decarboxylation of glycolate in the glycolate pathway. This excess CO_2 serves as the ultimate Hill oxidant instead of O_2 which can be reduced to toxic, active oxygens such as H_2O_2, O_2^-, and OH· (4). Removal of the excess energy can also be accomplished by synthesizing glycolate in the Calvin cycle (11).

On the other hand, detailed information on the synthesis and metabolism of glycolate in algae and *Euglena*, a photosynthetic protozoon, has not been available (4). This may be due, in part, to the fact that extensive CO_2 evolution takes place during glycolate metabolism (14, 19, 32) by a still unclarified mechanism and a considerable amount of glycolate is excreted into the medium as it is formed in the cells (18, 26).

In the present studies, experiments were designed to apply highly photorespiratory conditions, namely 100% O_2 atmosphere and illumination of 20,000 lux, to *E. gracilis* to force the organism to synthesize and metabolize glycolate at a maximum rate. The results should be valuable in contemplating the nature and function of photorespiration of *Euglena*.

MATERIALS AND METHODS

Cells. *E. gracilis* Z was cultivated photoautotrophically in air containing 5% CO_2 as described in a previous paper (31). Cells grown for 7 d were collected by centrifugation at 1,000*g* for 3 min at room temperature and used throughout experiments.

Determination of Excreted Glycolate. The *Euglena* cells were washed twice with distilled H_2O (27°C) and suspended in 10 mM K-phosphate (pH 6.8). The cell suspension (about 10^6 cells/ml) was bubbled with 100% O_2 at the rate of 50 ml/min per 20 ml of the suspension under illumination at 20,000 lux by two 40-w fluorescent lamps and a 300-w incandescent lamp at 27°C. After intervals, an aliquot of the suspension was removed, cooled promptly, and centrifuged at 3,000*g* for 2 min at 0°C. The supernatant, free from cells, was used for determination of excreted glycolate by the method reported previously (32).

Determination of Paramylon. *E. gracilis* was collected by centrifugation at 5,000*g* for 5 min and extracted three times with 2 ml each of 80% acetone. Protein was removed from the cells by suspending defatted cells in 2 ml of 1% SDS at 100°C for 5 min, and paramylon was sedimented by centrifugation at 10,000*g* for 10 min of the suspension. This procedure was repeated twice. Finally, precipitated paramylon was dissolved in 0.5 ml 1 N NaOH and then 1.5 ml 70% H_2SO_4 was added. Paramylon was determined by the phenol-H_2SO_4 method (7) using paramylon purified by Miyatake and Kitaoka (unpublished) as a standard.

Analysis of Amino Acid Pool. Cells (5×10^8) were extracted four times with 3 ml each of 0.45 N $HClO_4$. The combined extracts were passed through a column of Dowex 50 (H^+ form, 0.5 × 3 cm). The resin was washed with 50 ml distilled H_2O and amino acids were eluted with 20 ml 1 N aqueous ammonia. The eluate was evaporated to dryness and dissolved in 2 ml 0.2 M sodium citrate buffer (pH 2.2), and the solution was applied to an amino acid analyzer (Hitachi KLA-5).

Feeding Experiments of [1-^{14}C]Glycolate. *Euglena* cells which had been incubated for 12 h in the atmosphere of 100% O_2 under illumination at 20,000 lux in the phosphate buffer as described above were collected by centrifugation at 1,000*g* for 3 min and washed three times with distilled H_2O. The cells suspended in 10 mM K-phosphate (pH 6.8) were bubbled with 100% O_2 for 5 min in the dark. Feeding was started by the addition of 0.5 μCi of sodium [1-^{14}C]glycolate (7.2 μCi/μmol). The fed cells were illuminated at 20,000 lux in the presence of 100% O_2 at 27°C. Radioactivities in evolved CO_2 and cell components were determined according to the method described in the previous paper (32).

[1] This paper is the sixth in a series on the metabolism of glycolate in *Euglena gracilis*.

Determination of Chl. Chl was determined by the method of MacKinney (17).

RESULTS

Conversion of Paramylon to Excreted Glycolate. Figure 1 shows the time course of the excretion of glycolate when 5% CO_2-grown *E. gracilis* cells were transferred to a 100% O_2 atmosphere under illumination of 20,000 lux. The number of living cells and their motility were unchanged during 24 h of the experiments. The excretion proceeded linearly with incubation time for 8 h at a rate of 5.6 μmol/mg Chl·h, and then declined gradually. The concentration of glycolate in the incubation medium at the time after 10 h of incubation was 5.4 mM, which corresponds to 115 μg glycolate per 10^6 cells or per 310 μg of dry weight of original cells.

Excretion of glycolate from *E. gracilis* was almost completely inhibited by the addition of 50 μM DCMU, 20 mM bicarbonate, 10 mM monoiodoacetate, or 1 mM KCN. DCMU is an inhibitor of photosynthetic electron transfer (8, 14) and bicarbonate that of the oxygenase reaction of RuBPCO[2] (15). Monoiodoacetate and KCN are inhibitors of RuBPCO (21). Monofluoroacetate (10 mM), a potent inhibitor of the tricarboxylic acid cycle (9), showed no inhibition of the glycolate excretion, but $^{14}CO_2$ liberation was suppressed by 35% when *Euglena* cells were incubated with 0.1 μCi of [U-^{14}C]glucose (321 μCi/μmol) in the presence of the same concentration of monofluoroacetate. Under illumination at 20,000 lux, the rate of the glycolate excretion in ordinary air was one-fifth of that in 100% O_2. No glycolate was excreted in N_2. The excretion rate reached a constant level with 10,000 lux of light intensity in a 100% O_2 atmosphere.

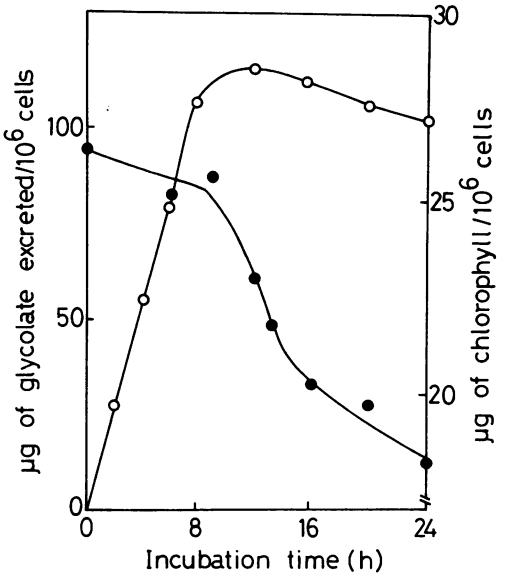

FIG. 1. Glycolate excretion and Chl degradation in 100% O_2 atmosphere under illumination at 20,000 lux in *Euglena*. The *Euglena* cells (2.3 × 10^6/ml) were incubated in 100% O_2 atmosphere under illumination at 20,000 lux at 27°C. Glycolate excreted (○); Chl content (●).

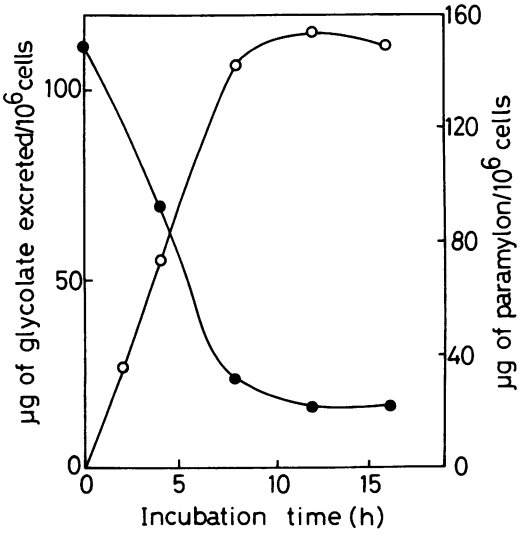

FIG. 2. Paramylon degradation during the glycolate excretion from *Euglena*. The *Euglena* cells (3.3 × 10^6/ml) were incubated as shown in the legend of Figure 1. Glycolate excreted (○); paramylon content (●).

Figure 1 also shows the Chl content in *Euglena* under a 100% O_2 atmosphere and 20,000 lux of light intensity. The content of Chl in the cells was nearly constant during the initial 8 h of the incubation, and then it began to diminish when the glycolate excretion ceased. The content of Chl after 24 h of incubation was 70% of the original content.

Figure 2 shows that the content of paramylon, the storage polysaccharide, in the 5% CO_2-grown cells of *Euglena* was decreased from 147 to 20 μg per 10^6 cells during the excretion of glycolate in a 100% O_2 atmosphere under illumination at 20,000 lux. Thus, the decrease of 127 μg paramylon per 10^6 cells was accompanied by the excretion of 115 μg glycolate per 10^6 cells. The glycolate excretion ceased when paramylon was exhausted. Cellular contents of protein and lipid, measured by the method of Yokota *et al.* (30), did not show changes during the experiment.

The 5% CO_2-grown *E. gracilis* cells (3 × 10^6/ml) were incubated for 12 h in a 0% $10O_2$ atmosphere under illumination at 20,000 lux to make paramylon be used up, and then washed with distilled H_2O. The cells were incubated with 2 mM [U-^{14}C]glucose (0.05 μCi/μmol) with bubbling of O_2 under illumination at 20,000 lux. Excreted glycolate was isolated and determined. The amounts of labeled glycolate produced from labeled glucose and excreted were 11.3 and 17.5 pmol/10^6 cells after 1 and 2 h, respectively, of the incubation. The rate of the conversion from glucose into excreted glycolate was 0.43 nmol/mg Chl·h.

Metabolism of Glycolate under 100% O_2 and 20,000 Lux of Illumination. Table I shows the cellular content of amino acids involved in the glycolate pathway in *E. gracilis* kept in a 100% O_2 atmosphere at 20,000 lux for up to 12 h. If the ordinary glycolate pathway as known in green plants were operative, the amounts of glycine and serine would be increased several-fold while glutamate decreased (6). No significant changes were seen in the contents of these amino acids during the incubation in which an extensive glycolate excretion took place. No ninhydrin-positive compound was detected in the incubation medium, indicating that none of the amino acids was excreted or leaked.

When 5% CO_2-grown *Euglena* was incubated in 100% O_2 under illumination at 20,000 lux for 12 h till the cells cease to excrete glycolate, washed with distilled H_2O and fed [1-^{14}C]glycolate

Table I. *Effect of 100% O_2 and Light of 20,000 Lux on Amino Acid Pool*

The *Euglena* cells (1.5 × 10^6/ml) were incubated as in Figure 1.

Amino Acid	Amount of Amino Acid after Incubation		
	0 h	4 h	12 h
	nmol/10^6 cells		
Serine	0.81	1.01	0.66
Glutamate	0.72	0.74	1.22
Glycine	0.96	0.93	1.01

[2] Abbreviation: RuBPCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Table II. *Distribution of Radioactivity of [1-^{14}C]Glycolate under 100% O_2 and 20,000 Lux Conditions in Euglena*

The *Euglena* cells (4.6×10^6/ml) were preincubated under conditions described in Figure 1 for 12 h. After washing the cells with distilled H_2O the cells were fed for 3 min with [1-^{14}C]glycolate in the same cell density as in the preincubation.

Fraction	Radioactivity	Total Radioactivity Taken up
	cpm	%
Evolved CO_2	8,955	87.4
Sugar-acid	711	6.9
Amino acid	215	2.1
Paramylon	204	2.0
Protein	160	1.6
Lipid	0	0

under the same conditions, the labeled glycolate was taken up and metabolized actively (Table II). Evolved CO_2 accounted for 87% of the total radioactivity taken up and the sugar-acid fraction only 7%. Incorporation into amino acid and protein fractions was almost negligible. The same results were obtained when the cells were incubated for 5 min.

DISCUSSION

In a 100% O_2 atmosphere under illumination at 20,000 lux, the rate of the excretion of glycolate from *E. gracilis* was 5.6 μmol/mg Chl·h (Fig. 1). The amount of excreted glycolate accounted for about 30% of dry weight of the *Euglena* cells after incubation for 12 h. The excretion was completely inhibited by monoiodoacetate, indicating that glycolate is synthesized through the reaction of RuBPCO in the Calvin cycle, as previously postulated in *Euglena* and other photosynthetic organisms (1). Levels of the intermediates of the Calvin cycle should fall as the result of synthesis and excretion of a large amount of glycolate; the excretion of glycolate proceeded linearly with time for at least 8 h after the start of bubbling the incubation mixture with O_2 under illumination at 20,000 lux, and was accompanied by decrease of the paramylon content (Fig. 2). Other cell components did not show any change in their contents. These results indicate that paramylon carbon is used for the synthesis of glycolate. Exogenous [U-^{14}C]glucose was also converted to excreted, labeled glycolate; the rate of the conversion was much lower than that from intracellular paramylon, probably due to low uptake activity of exogenous glucose in autotrophic *Euglena* (20). Such conversion of reverse polysaccharide to glycolate has been postulated in other organisms (5, 12, 16). Cessation of the glycolate excretion followed exhaustion of the polysaccharide in *E. gracilis*. After the stop of the glycolate excretion, the Calvin cycle can not use light energy for any purpose in 100% O_2, and NADPH should accumulate in chloroplasts. Such a condition should allow chloroplasts to reduce readily O_2 to toxic, active oxygens which destroy chloroplasts (2). Chl degradation was actually observed after the cessation of the glycolate excretion (Fig. 1).

The glycolate excretion from *Euglena* was not affected by the addition of monofluoroacetate, indicating that the flow of paramylon carbon to glycolate is not associated with the tricarboxylic acid cycle; glycolate is not synthesized from CO_2 refixed photosynthetically after the complete oxidation of paramylon to CO_2 through the cycle. Inasmuch as chloroplasts have active triose-P translocator in their envelopes (27), paramylon carbon may enter the chloroplasts after being converted to triose phosphate or 3-P-glycerate, common intermediates of glycolysis and the Calvin cycle, and then be converted to glycolate in the cycle. Bicarbonate was not required for the synthesis and excretion of glycolate and in fact inhibited the synthesis of glycolate in *Euglena* in the present experiment, unlike in *Chlorella* and *Scenedesmus* (5).

During the incubation of the *Euglena* cells in an atmosphere of 100% O_2 under illumination at 20,000 lux, 127 μg per 10^6 cells of paramylon disappeared and 115 μg per 10^6 cells of glycolate was excreted (Fig. 2). Inasmuch as the percentage composition of carbon in paramylon is 42.9, 127 μg paramylon contains 54.5 μg carbon. Carbon occupies 31.6% of the weight of glycolic acid, and therefore 115 μg of the acid corresponds to 36.6 μg carbon. Accordingly, the results summarized in Figure 2 show that 67% of paramylon carbon which diminished during the incubation was transformed to excreted glycolate at the rate of 5.6 μmol/mg Chl·h. The remaining paramylon carbon must have been metabolized through glycolate at a rate of 2.8 μmol/mg Chl·h. The excreted glycolate may represent the part of glycolate synthesized at a rate higher than the rate of the glycolate metabolism. This view may be supported by the fact that the rate of the metabolism of glycolate estimated above is close to the activity of glycolate dehydrogenase (3.0 μmol/mg Chl·h) of the same *Euglena* cells (31).

In photoheterotrophically grown *Euglena* (32), glycolate is transformed to glycine and serine with concomitant decrease of glutamate in the amino acid pool in ordinary atmosphere. The linear increase of glycine pool with increasing concentration of O_2 has been reported in soybean leaves (6). In the present experiments under highly photorespiratory conditions, however, the metabolism of glycolate did not cause decrease of glutamate and increases of glycine and serine in the amino acid pool (Table I). The carboxyl carbon of added glycolate was not incorporated into the latter two amino acids but was decarboxylated (Table II). As we have previously postulated, the carboxyl carbon of glycolate is liberated as CO_2 at the step of glyoxylate in the glycolate pathway in *Euglena* (29, 32). *Anacystis* decarboxylates 86% of the carboxyl carbon of glycolate taken up and that the liberation of CO_2 is not affected by an inhibitor of the conversion of glycine to serine (19). The mechanism of the glyoxylate decarboxylation in *E. gracilis* will be published elsewhere. After the decarboxylation, carbon from glycolate was not refixed by photosynthesis (Table II). Decarboxylation of glyoxylate in the glycolate pathway is apparently related to the dissipation of excess light energy under the present experimental conditions (Fig. 3).

Figure 3 is a schematic illustration of the metabolism of glycolate in 5% CO_2-grown cells of *Euglena* placed in 100% O_2 under illumination at 20,000 lux. Glycolate is synthesized from RuBP in the Calvin cycle, accompanied by consumption of NADPH. Photosynthetic intermediates depleted by the synthesis of glycolate are replenished by paramylon carbon, probably via triose-P or 3-P-glycerate. Two-thirds of synthesized glycolate is excreted and the remaining one third oxidized in mitochondria (10). Most of glyoxylate, the primary oxidation product of glycolate, is decarboxylated by the reaction with H_2O_2 produced by a Mn^{2+}-dependent NADPH oxidase in chloroplasts (Yokota and Kitaoka, unpublished). The aldehyde carbon of glyoxylate is reported to be utilized for the synthesis of C_1 units after decarboxylation (10). A part of glyoxylate may also be rereduced to glycolate by the glycolate-glyoxylate shuttle which functions in mitochondria of *Euglena*, serving for the disposal of excess cellular NADPH (28). Inasmuch as Chl in the present experiments started degradation as soon as paramylon was exhausted and the supply of carbon for the synthesis of glycolate stopped, it seems that the synthesis and oxidative decomposition of glycolate, but not operation of the whole glycolate pathway metabolism, chiefly contributes to dissipation of excess light energy captured by *Euglena* chloroplasts. The incomplete metabolism of glycolate in *E. gracilis* in a high O_2 atmosphere under illumination of high light intensity (Fig. 3), may be an example of the "primitive photorespiration," proposed by Lloyd *et al.* (13). Algae, which have the machinery for the glycolate metabolism in mitochondria, should also follow this picture.

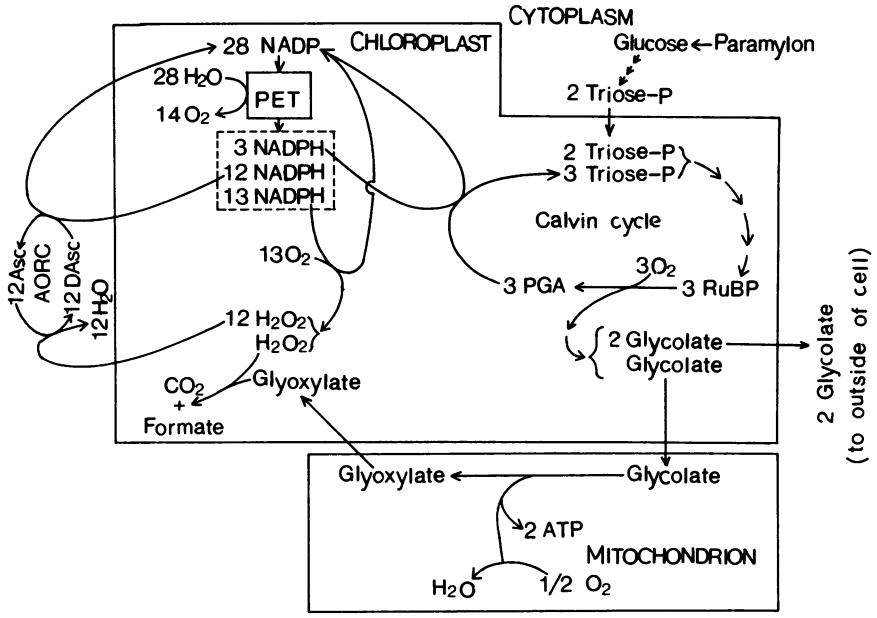

FIG. 3. Carbon and energy metabolism in *Euglena* under highly photorespiratory conditions. Asc, ascorbate; DAsc, dehydroascorbate; AORC, ascorbate oxidation-reduction cycle; PET, photosynthetic electron transfer chain: PGA, 3-phosphoglycerate; RuBP, ribulose-1,5-bisphosphate; Triose-P, triosephosphate.

To what extent can the synthesis and metabolism of glycolate dissipate excess light energy in *Euglena*? The rate of the synthesis of glycolate, about 10 μmol/mg Chl·h, in the absence of CO_2 and presence of 100% O_2 at a saturating light intensity corresponds to the sum of the rates of the excretion and metabolism of glycolate. Inasmuch as the synthesis of 1 mol glycolate through the Calvin cycle requires 1 mol NADPH, the rate of the synthesis is equal to that of NADPH consumption. On the other hand, *Euglena* converts light energy to chemical energy in the photosynthetic CO_2 fixation at the rate of 60 μmol/mg Chl·h in the presence of saturating CO_2 and light (24). This rate corresponds to the utilization rate of NADPH at 120 μmol/mg Chl·h. These considerations imply that the synthesis and metabolism of glycolate in *Euglena* is able to consume less than one-tenth of light energy (NADPH) captured by this organism in the absence of CO_2. The remaining NADPH must be largely reoxidized, probably by L-ascorbate peroxidase-relating L-ascorbate oxidation-reduction cycle in cytoplasm (22, 23), as shown in Figure 3. NADPH may also be used to reduce and decompose glyoxylate. However, because cessation of the synthesis of glycolate after exhaustion of paramylon promptly caused Chl degradation (Fig. 1), the synthesis and metabolism of glycolate apparently functions critically in the dissipation of excess light energy in *Euglena*.

Such a function of the synthesis and metabolism of glycolate is expected to be present in 5% CO_2-grown *Euglena* under illumination at a high light intensity, even if this organism is transferred to ordinary air. Photosynthetic microorganisms grown on high CO_2 concentration have a low CO_2 fixation rate in air (3). If these cells are illuminated at a high light intensity in the presence of ordinary air, excess NADPH will be utilized for the fixation of O_2 in the Calvin cycle, namely for the synthesis of glycolate. In fact, *Euglena* grown on 5% CO_2 excreted glycolate at the rate of 1 μmol/mg Chl·h in ordinary air under illumination at 20,000 lux in the present experiments. Inasmuch as the cells can metabolize glycolate at the rate of 3.0 μmol/mg Chl·h (31), the rate of the synthesis of glycolate under such conditions will be 4.0 μmol/mg Chl·h. The decreased rate of the glycolate synthesis may be a result of the competition for light energy between the glycolate synthesis and the CO_2 fixation in the Calvin cycle in ordinary air. Under such conditions, namely in ordinary air and at high light intensity, light energy must be shared among the synthesis of glycolate, the CO_2 fixation and the ascorbate system.

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