

Glycolate Pathway In Algae¹

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Summary. No glycolate oxidase activity could be detected by manometric, isotopic, or spectrophotometric techniques in cell extracts from 5 strains of algae grown in the light with CO₂. However, NADH:glyoxylate reductase, phosphoglycolate phosphatase and isocitrate dehydrogenase were detected in the cell extracts. The serine formed by *Chlorella* or *Chlamydomonas* after 12 seconds of photosynthetic ¹⁴CO₂ fixation contained 70 to 80 % of its ¹⁴C in the carboxyl carbon. This distribution of label in serine was similar to that in phosphoglycerate from the same experiment. Thus, in algae serine is probably formed directly from phosphoglycerate. These results differ from those of higher plants which form uniformly labeled serine from glycolate in short time periods when phosphoglycerate is still carboxyl labeled.

In glycolate formed by algae in 5 and 10 seconds of ¹⁴CO₂ fixation, C₂ was at least twice as radioactive as C₁. A similar skewed labeling in C₂ and C₃ of 3-phosphoglycerate and serine suggests a common precursor for glycolate and 3-phosphoglycerate. Glycine formed by the algae, however, from the same experiments was uniformly labeled.

Manganese deficient *Chlorella* incorporated only 2 % of the total ¹⁴CO₂ fixed in 10 minutes into glycolate, while in normal *Chlorella* 30 % of the total ¹⁴C was found in glycolate. Manganese deficient *Chlorella* also accumulated more ¹⁴C in glycine and serine.

Glycolate excretion by *Chlorella* was maximal in 10 mM bicarbonate and occurred only in the light, and was not influenced by the addition of glycolate. No time dependent uptake of significant amounts of either glycolate or phosphoglycolate was observed. When small amounts of glycolate-2-¹⁴C were fed to *Chlorella* or *Scenedesmus*, only 2 to 3 % was metabolized after 30 to 60 minutes. The algae were not capable of significant glycolate metabolism as is the higher plant.

The failure to detect glycolate oxidase, the low level glycolate-¹⁴C metabolism, and the formation of serine from phosphoglycerate rather than from glycolate are consistent with the concept of an incomplete glycolate pathway in algae.

During ¹⁴CO₂ photosynthesis by leaves of higher plants, glycolate, glycine, and serine are rapidly labeled with ¹⁴C. From many investigations involving the rate of labeling, the percent distribution of ¹⁴C products which are formed from added glycolate-¹⁴C, and the isolation of specific enzymes, a glycolate pathway has been proposed as a metabolic route for part of the newly fixed carbon during photosynthesis (30). In this pathway, phosphoglycolate is hydrolyzed to free glycolate, which is oxidized to glyoxylate via glycolate oxidase. The subsequent amination of glyoxylate to glycine and its conversion to serine allow for the formation

of glycerate and the ultimate formation of sugars in the light or malate in the dark.

For several reasons it has been assumed that algae also contained a glycolate pathway similar to that in the higher plant. Algae rapidly labeled glycolate, glycine, and serine during ¹⁴CO₂ photosynthesis (3, 5, 21). Further, it has been reported that glycolate-¹⁴C was converted by *Scenedesmus* to glycine and serine (24). It was known that higher plants, rapidly convert glycolate-1-¹⁴C to both glycine-1-¹⁴C and serine-1-¹⁴C and convert glycolate-2-¹⁴C to glycine-2-¹⁴C and serine-2, 3-¹⁴C (22, 31, 34, 35).

Our results are not consistent with the assumptions noted above, and we have concluded that the glycolate pathway is not complete in certain algae if they are grown in the light with CO₂. Thus glycolate metabolism represents a metabolic difference between higher plants and algae. A gly-

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colate oxidase typical of the higher plant has not been detected in any algae, although other enzymes of the glycolate pathway are present. *Chlorella* and *Chlamydomonas* do not accumulate glycolate in the presence of the hydroxymethanesulfonate inhibitors of glycolate oxidase (32). The initial ^{14}C -labeling pattern in serine formed during algal $^{14}\text{CO}_2$ photosynthesis indicate that serine is formed from 3-P-glycerate rather than glycolate. These findings are consistent with the excretion of glycolate by algae rather than its metabolism (33).

Experimental Procedure

Algae. Stock cultures were obtained from the University of Indiana collection. Three algae strains, *Chlorella pyrenoidosa* Chick, number 393; *Chlamydomonas reinhardtii*, Dangeard (-), number 90; and *Ankistrodesmus braunii* (Naeg.) Collins, number 245 were routinely used in all of the work. In some experiments *Scenedesmus obliquus* (Gaffron D-3), number 393 and *Chlorella pyrenoidosa* (Warburg) (from Mary Stiller, Purdue University) were also used. *Chlorella*, *Ankistrodesmus*, and *Scenedesmus* were grown in medium V of Norris et al. (18) at 20°, *Chlamydomonas* in a phosphate rich medium (21) at 20°, and *Chlorella* (Warburg) in Warburg medium K as modified by Stiller (26). All the algae were cultured continuously in 3 liter Fernbach flasks on a reciprocating Eberbach shaker in plant growth chambers which provided about 1000 ft-c of light to the surface of the algal medium. The cultures were continuously aerated with a mixture of 0.3 to 0.5 % CO_2 in air. Algae were harvested when they were in a stage of logarithmic growth which was achieved by innoculating 1400 ml of fresh sterile nutrient with 100 ml of algal culture. Algae were centrifuged from the medium at $1000 \times g$ at 5°, and washed once with water. Finally, the cells were resuspended in water or in 1 mM phosphate buffer at a designated pH.

Manganese deficient cultures of *Chlorella pyrenoidosa* Chick were maintained on the inorganic salt medium of Norris et al. (18) without MnCl_2 added to the micro-nutrients. The Mn-deficient medium was prepared with double-distilled deionized water, and the chemicals were recrystallized in similar water and analyzed for Mn^{++} .

Sonication of Algae. After harvesting the algae from the culture medium, the packed cell volume was diluted to a 30 % (v/v) suspension in a designated medium. The cells were ruptured by a 125 watt Branson Sonifier (Heat Systems, Melville, N.Y.). A 15 ml suspension of algae at pH 7.5 to 8.5 in a 25 ml rosette cell (supplied by Heat Systems) was cooled in an ethanol-water-ice bath at -5°. The regular tip of the sonicator was positioned as close to the bottom of the cell as possible, and the instrument operated at maximum output. The Sonifier ruptured 90 % of the *Chlamy-*

domonas cells after 5 minutes, but only 60 to 70 % of the *Chlorella* or *Ankistrodesmus* cells after 10 to 15 minutes. The apparent turbidity of the suspension decreased as the cells were broken and particle size decreased. The pH of the broken suspension decreased during sonication in water from 6.5 to 5.8, in 50 mM phosphate from pH 8.3 to 7.5, and in 200 mM cacodylate buffer at pH 6.3 there was no pH change. At a pH of less than 5.5 the ruptured suspension would coagulate and greatly reduce the efficiency of the sonication. After sonication, the suspensions were centrifuged at $3.5 \times 10^4 \times g$ for 10 minutes. The clear, dark-green supernatant was used as a cell extract for enzyme assays or isolation.

Enzyme Assays. Glycolate oxidase was measured by 3 procedures. The standard manometric assay was run in a final concentration of 20 mM phosphate at pH 8.3, 0.1 mM FMN and 3.3 mM glycolate. In a spectrophotometric assay the increased absorbance due to the formation of glyoxylate phenylhydrazone was measured at 324 μm (10). This reaction mixture contained in a total volume of 3 ml, 2 ml of 0.1 M phosphate pH 8.3, 0.1 ml of 0.1 M phenylhydrazine hydrochloride adjusted to pH 6.8, 0.1 ml of 0.1 M cysteine, 0.3 ml of 0.1 M glycolate and 0.5 ml enzyme (cell extract). In the very sensitive isotopic assay, a known amount of glycolate-2- ^{14}C was added to the crude cell sonicate either before or after centrifugation. The mixture was incubated with shaking and aliquots were removed and mixed with hot methanol at time intervals. Glycolate metabolism was measured by disappearance of ^{14}C and by 2 dimensional chromatography of the reaction mixture for the identification of products.

Glyoxylate reductase was measured by the oxidation of reduced pyridine nucleotide at 340 μm after the addition of glyoxylate (40). The assay mixture contained 1 ml 0.1 M phosphate at pH 6.5, 0.5 ml of 0.1 M glyoxylate, 0.05 ml of 0.02 M NADH, 0.1 ml cell extract, and water to 3 ml.

Phosphoglycolate phosphatase assay has been described (1, 23). The reaction mixture contained 0.1 ml of 10 mM cacodylate buffer at pH 6.3 and 20 mM MgSO_4 , 1.0 ml of 10 mM P-glycolate at pH 6.3 and 1.0 ml enzyme adjusted to pH 6.3. The mixture was incubated at 30° after the addition of the enzyme and the reaction stopped with 1.0 ml of 10 % trichloroacetic acid. After centrifugation, 1 ml of the supernatant fluid was assayed for phosphate.

Isocitrate dehydrogenase was measured by the procedure described by Syrett et al. (27). The assay followed the rate of NADP reduction and the reaction mixture contained 1.0 ml of 0.1 M phosphate at pH 7.5, 1.0 ml 50 mM MgCl_2 , 0.1 ml of 60 mM cysteine, 0.05 ml of 10 mM NADP, 0.05 ml cell extract; the reaction was initiated with 0.1 ml of 0.1 M isocitrate.

Isotopic Procedure. Glycolate-2- ^{14}C and phosphoglycolate-2- ^{14}C were purchased from Nuclear Research Chemicals, Orlando, Florida, and were determined by us on 2 dimensional paper chromatograms (4) to be chromatographically pure. The photosynthetic experiments with $^{14}\text{CO}_2$, the isolation of the ^{14}C products, and the degradation procedures for 3-P-glycerate, glycine glycolate and serine have been described elsewhere (8, 12, 42). Each time period listed in table I represent a separate experiment, and at least 2 degradations were performed on each compound. At least 3000 cpm of glycerate or serine were used with unlabeled carrier for each degradation: 500 to 1000 cpm of glycolate or glycine were used.

Metabolism studies of glycolate- ^{14}C or P-glycolate- ^{14}C by whole algae were run in a 15 ml lollipop similar to that used for $^{14}\text{CO}_2$ fixation experiments (32). Aliquots were removed and filtered with vacuum in about 10 seconds on either a 25 mm Millipore filter with $0.8\ \mu$ pores or on a 5 to 10 mm thick pad of celite over a sintered glass filter. A small amount of celite was sometimes necessary to avoid clogging of the Millipore filters. After filtration the Millipore filter was not washed, but the thicker celite filter pad with cells was washed with 1 volume of medium. Then the algal cells were extracted consecutively with excess hot methanol, hot water, and then hot ethanol again until no green color remained on the filter. Aliquots of both the medium and the cell extracts were counted for total ^{14}C recovery and percent distribution of ^{14}C between the medium and cells; other aliquots were chromatographed (4) for product analysis.

Results

Glycolate Oxidase. There are no literature citations to this enzyme from algae except for 3 preliminary reports (14, 19, 39) which suggest that if the oxidase were present, it is likely different from that of higher plants. Also, Okuda and Shoji (20) have reported that glycolate stimulates the respiration of nitrogen starved *Chlorella*. They suggested that incorporation of glycolate into amino acids via the glycolate pathway was related to this respiratory stimulation. We have attempted to isolate and characterize glycolate oxidase from sonicated preparations of each of the 5 algae cited in the methods section. No glycolate oxidase activity was observed from any of the algal strains by any of the assay procedures described below. Cells of 2 or 3 day old cultures in random, logarithmic growth were generally used. Before sonication the algae were suspended either in 0.1 M phosphate buffer at pH 8.3 or 6.3, in 0.1 M phosphate at pH 8.3 containing 20 mM Na-glycolate with or without 0.1 mM FMN, or in 0.1 M phosphate at pH 8.3 containing 20 mM cysteine with or without 20 mM Na-glycolate. After sonication part of

each suspension was centrifuged. The original crude extract, the supernatant fluid after centrifugation and the residue were each assayed manometrically at 25°. No glycolate oxidase activity was observed after 60 minutes of assay. When glycolate-2- ^{14}C was added to extracts from *Chlorella pyrenoidosa* (Chick), and *Chlamydomonas*, it was recovered as glycolate- ^{14}C and less than 1% or none of the label after incubation at 25° for 30 minutes was lost or converted to glycine, serine, or any compound which could be detected by paper chromatography. Since one of the predicted products, glyoxylate, can not be detected quantitatively by the paper chromatography procedures employed, the formation of glyoxylate phenylhydrazone (10) was followed to confirm the other assays. This assay also did not indicate any glycolate conversion to glyoxylate by any of the algal extracts prepared from cells in random cultures grown in continuous light.

In the above experiments several controls were run. A) When glycolate oxidase, prepared from tobacco leaves by $(\text{NH}_4)_2\text{SO}_4$ fractionation (9), was added to extracts from *Chlorella*, no inhibition of its activity was observed. B) Addition of the glycolate oxidase preparation from tobacco leaves to *Chlorella* cell suspensions before sonication resulted in a maximum of only 10% reduction of the oxidase activity during the sonication procedure. C) Baker and Tolbert (2) have isolated a labile form of glycolate oxidase by grinding leaves with 20 mM substrate, but this procedure did not yield a glycolate oxidase in algae. D) Increasing the partial pressure of oxygen in the assays did not promote any activity. E) Dialysis to remove an inhibitor did not activate a glycolate oxidase. F) $(\text{NH}_4)_2\text{SO}_4$ fractionation of *Chlorella* extracts, according to the procedure for preparing glycolate oxidase from leaves (9), did not produce an enriched protein fraction with activity. G) The detection of isocitrate dehydrogenase and other enzymes demonstrated that active enzymes were present in the sonicated cell-free algal extracts.

Isocitrate Dehydrogenase. Our sonicated extracts from *Chlorella pyrenoidosa* (Chick) catalyzed NADP reduction at a rate of 30 μ moles per 10 minutes per 0.1 ml of extract which was comparable to that reported by Syrett et al. for *Chlorella vulgaris* (27). This activity depended on isocitrate and was specific for NADP.

Glyoxylate Reductase. This enzyme in algal extracts was dependent upon NADH but not NADPH. A 0.5 ml aliquot of *Chlorella* extract oxidized 6 μ moles NADH per minute with the glyoxylate substrate. The extracts were also capable of reducing hydroxypyruvate with NADH. The glyoxylate reductase from spinach leaves and D-glycerate dehydrogenase from tobacco leaves have approximately the same K_m values for hydroxypyruvate and glyoxylate (15, 40). Thus, glyoxylate and hydroxypyruvate reductase and D-glycerate

Table I. Percent Distribution of ^{14}C in Phosphoglycerate, Glycolate, Glycine, and Serine Formed by Algae During Photosynthesis

Fifty ml of 1% suspension of algae in 2 mM phosphate buffer at pH 6.5 were exposed in a lollypop at 20° to 3000 ft-c of light. Each time period represented a different experiment. At zero time 100 μc of $\text{NaH}^{14}\text{CO}_3$ was added to give a final concentration of about 0.2 mM. Products were isolated by paper chromatography and degraded (see Methods).

Time sec	Percent distribution of ^{14}C in products										
	P-Glycerate			Serine			Glycolate		Glycine		
	C ₁	C ₂	C ₃	C ₁	C ₂	C ₃	C ₁	C ₂	C ₁	C ₂	
	<i>Chlorella pyrenoidosa</i> (chick)										
7	84	4	12	78	6	16	
10	35	65	
12	82	3	15	80	4	16	50	50	
20	43	57	
60	47	53	
	<i>Chlorella pyrenoidosa</i> (Warburg)										
5	96	1	3	27	73	
10	93	2	5	39	61	
	<i>Chlamydomonas reinhardtii</i>										
12	79	4	17	69	7	24	30	70	49	51	
20	42	58	
60	48	52	
	Tobacco leaves*										
11	65	16	19	40	39	21	52	48	49	51	

* From Hess and Tolbert (12).

dehydrogenase were both present or one enzyme was acting on both substrates.

Phosphoglycolate Phosphatase. Extracts from all the algae contained active P-glycolate phosphatase. In sonicated extracts from *Chlorella* a ratio of 20:1 was obtained with respect to the rate of hydrolysis of P-glycolate to P-glycerate. This 20:1 ratio indicated that algal extracts had a greater specificity for P-glycolate than did the partially purified P-glycolate phosphatase from tobacco or wheat leaves which gave a P-glycolate:P-glycerate ratio of 3:1 after an acetone fractionation of the crude sap (23,38). P-glycolate- ^{14}C was rapidly and completely hydrolyzed to glycolate- ^{14}C by algal extracts.

Formation of Carboxyl Labeled P-Glycerate and Serine. The absence of glycolate oxidase in algae (see previous section) raised speculation about ^{14}C distribution in glycine and serine which, in higher plants, are primarily formed from uniformly labeled glycolate. Therefore, the ^{14}C distribution was determined in 3-P-glycerate, glycine and serine which had been formed by *Chlamydomonas* and *Chlorella* during short periods of $^{14}\text{CO}_2$ fixation. Both 3-P-glycerate and serine were similarly carboxyl labeled (table I) and both had similar skewed labeling for carbons 2 and 3 in which C₃ was at least 2 times more active than C₂. In earlier experiments (7,11) skewed labeling patterns in C₂ and C₃ of 3-P-glycerate were not pronounced. The reason for this is not known. One experimental difference may be in the lower levels of CO_2 used in our experiments and the amount of glycolate formed.

The formation of carboxyl labeled serine by algae differs markedly from the formation of

uniformly labeled serine by tobacco leaves (table I) or by several other plants (22) in equally short periods of time when 3-P-glycerate was still carboxyl labeled. In the algal experiments serine became uniformly labeled at about the same rate as did 3-P-glycerate.

The Formation of Asymmetric Glycolate. In higher plants glycolate has been found to be uniformly labeled during initial $^{14}\text{CO}_2$ fixation times (7,12,22). It was assumed that glycolate formed by algae would also be uniformly labeled, although these data have not been reported. We found nearly uniformly labeled glycolate to be present after 20 seconds of photosynthesis. However, at 5 and 10 seconds the hydroxyl carbon or C₂ of glycolate was at least twice as labeled as the carboxyl carbon (table I). This skewed labeling in glycolate was formed by algae at low bicarbonate concentration and was somewhat similar to the distribution of ^{14}C in the second and third carbons of P-glycerate and serine. These data indicate that glycolate and carbons 2 and 3 of P-glycerate and serine may have a common precursor. This fact supports the hypothesis that glycolate is probably produced from carbon atoms 1 and 2 of pentose phosphates of the photosynthetic carbon reductive cycle. The labeling in glycolate indicate that it could also arise from C₂ and C₃ of 3-P-glycerate via hydroxypyruvate. These hypothetical routes for glycolate formation during photosynthesis vary only in the precursor of the C₂-thiamine pyrophosphate. However if glycolate were produced from 3-P-glycerate, its synthesis would occur with no net CO_2 fixation, while if the C₂-precursor were a sugar phosphate of the photosynthetic carbon cycle,

sustained net CO₂ fixation would be possible during glycolate biosynthesis.

Formation of Uniformly Labeled Glycine During ¹⁴CO₂ Fixation. The glycine formed by the algae was uniformly labeled (table I), and this result must be compared with the formation of carboxyl labeled serine and α-labeled glycolate in the same experiment. Chang and Tolbert also observed that glycine formed by chloroplasts was uniformly labeled (8). Zak reported that glycine formed by *Chlorella* after 13 seconds photosynthesis had 49% of its ¹⁴C in the C₁, while for the same length of time serine contained 82% of its ¹⁴C in the carboxyl group (37). These labeling patterns eliminate the possibility that uniformly labeled glycine was formed from carboxyl labeled serine via the trans-hydroxymethylase reaction. The skewed labeling pattern of glycolate, the absence of glycolate oxidase, and the absence of glycolate-¹⁴C metabolism by the algae are inconsistent with glycine formation from glycolate. Thus the pathway for rapid labeling of glycine by algae is uncertain.

Manganese Deficient Chlorella. After 20 days, with nutrient changes every third day, a highly significant reduction of the growth rate of *Chlorella pyrenoidosa* (Chick) was observed with the Mn-deficient medium (fig 1). The slower growth rate with the deficient culture could be restored to the normal rate in 24 hours by adding an appropriate amount of MnCl₂ to the deficient medium. Since the growth rates were measured on cultures with similar initial cell population, the Mn-deficient rate indicates a slower log phase of growth.

The percent distribution of ¹⁴C among the products of photosynthetic ¹⁴CO₂ fixation were examined for normal and Mn-deficient *Chlorella* (table II). The nearly linear rate of total fixation for either culture over the 10 minute period indicated that the ¹⁴CO₂ concentration did not become a limiting factor. Glycolate-¹⁴C accumulation in the Mn-deficient culture was markedly reduced but not eliminated. Formation of ¹⁴C labeled glycine and serine by the Mn-deficient algae increased. These results confirm an earlier report on the manganese requirement for glycolate formation during photosynthesis by *Chlorella* (28). Tamer, et al. (28) also reported an increased amount of ¹⁴C labeling for glycine and serine by Mn-deficient *Chlorella*.

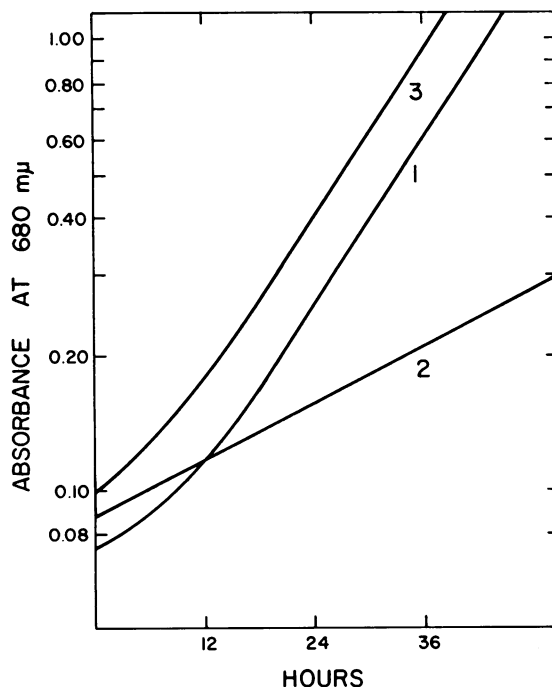


FIG. 1. Growth rates for cultures of *Chlorella pyrenoidosa* (Chick). Curve 1: Normal *Chlorella* culture. Curve 2: Mn-deficient culture. Curve 3: Mn-deficient culture with MnCl₂ added.

Glycolate Excretion and Reabsorption. The excretion of glycolate by algae has been well documented (17, 33, 36, 37), and we have observed glycolate loss from chloroplasts (12, 29). No metabolic function has yet been established for this phenomenon. Maximum glycolate excretion occurs at low CO₂ concentrations (0.1–0.3%), high light intensity, and high oxygen partial pressure (for review see ref. 30). The excretion of glycolate has been measured in algal supernatant media as ¹⁴C-glycolate or by the colorimetric procedure of Calkins for glycolate (6). Since the cells were washed once with water and resuspended in dilute buffer, the colorimetric assay for glycolate in the supernatant fluid was quantitative for added glycolate and controls without glycolate gave nearly zero readings. All measurements with variable

Table II. Percent Distribution of ¹⁴C Fixed by *Chlorella pyrenoidosa* (Chick)

Time (mins)	Normal control			Mn-deficient		
	1	3	10	1	3	10
Total fixation (c/m × 10 ⁻⁴ /ml algae)	4.6	20.1	35.6	4.5	17.9	46.2
Products	% Distribution					
Sugar phosphates	76.5	63.1	37.3	61.9	59.0	42.8
Glycolate	7.0	14.5	30.9	4.0	3.3	2.4
Glycine	2.6	5.2	5.2	7.1	6.9	5.7
Serine	4.3	9.5	10.1	13.5	15.8	20.4
Other	9.6	7.7	16.5	13.6	14.8	28.6

Table III. *Glycolate Excretion by Chlorella pyrenoidosa* (Chick)

Glycolate excreted by 2% *Chlorella* suspension in bicarbonate during photosynthesis in 3000 ft-c.

HCO ₃ ⁻ Conc	Glycolate in supernate
moles/l	μg/hr/ml
0	9
0.0001	9
0.001	25
0.01	78
0.1	10

CO₂ concentration were done in buffers at pH ranges of 4.5 to 6.5. Recently we reported that glycolate formation and excretion was less inhibited at high pH than the formation of other products of CO₂ fixation (21). Further investigation on glycolate excretion by algae in bicarbonate (pH 8.3) has revealed that at this pH maximum glycolate excretion occurs when the algae are in 10 mM NaHCO₃ (table III). In 10 mM NaHCO₃ glycolate excretion was linear during the first hour. When bicarbonate was used in the presence of 10 mM glycolate, a time-dependent excretion of glycolate occurred for at least 1 hour (column 1, table IV). The latter experiment was run with *Chlorella* grown in 5% CO₂ and air, and the results were nearly similar to those with *Chlorella* grown in 0.2% CO₂ and air. Thus, neither the presence of glycolate or NaHCO₃ in the supernatant fluid nor high CO₂ concentration during growth of the algae prevented glycolate excretion.

With randomized cultures of *Chlorella*, *Chlamydomonas*, *Ankistrodesmus*, and *Scenedesmus* in the log phase of growth we have observed no significant uptake of ¹⁴C labeled glycolate or P-glycolate during time periods of 10 to 120 minutes. Algal suspensions were incubated with 10⁻² to 10⁻⁷ M solutions of labeled substrates in 3000 ft-c of light or in dark, at pH 3.5 to 8.5 in air, oxygen or nitrogen, and without buffer or with phosphate or bicarbonate buffer. Typical results are presented

Table IV. *Excretion and Uptake of Glycolate by Chlorella*

A 2% *Chlorella* (grown in 5% CO₂) suspension was prepared in 10 mM sodium glycolate with either 10 mM NaHCO₃ or water. The initial glycolate concentration was 25 μg/ml as determined by the colorimetric assay.

Time	Net change from	Net change
	10 mM NaCO ₃ and 0.33 mM glycolate	from 0.33 mM glycolate
min	μg glycolate/ml	
0	0	0
5	4	2
15	9	-2
30	16	-5
60	39	2

in table V. Pretreatment of the algae for 1 hour in light or darkness and air or nitrogen did not result in subsequent glycolate metabolism. The small amount of activity remaining with the cells did not increase with time, and could have been caused by surface absorption of the labeled acids by the algae or filter. ¹⁴C did not disappear from the culture medium and chromatography of the medium indicated neither metabolism of glycolate nor hydrolysis of P-glycolate. Glycolate uptake from 10⁻⁴ and 10⁻⁵ M solutions were also determined by the quantitative colorimetric measurement (6) of the remaining glycolate in the algal medium, but no significant indication of glycolate disappearance was obtained (column 2, table IV).

Table V. *Uptake of Glycolate-2-¹⁴C or Phosphoglycolate-2-¹⁴C by Chlamydomonas*

A 2% *Chlamydomonas* suspension in 40 mM phosphate (initial pH 4.0) in 3000 ft-c white light.

Time	¹⁴ C in Algal cells	
	Glycolate-2- ¹⁴ C	P-glycolate-2- ¹⁴ C
min	%	%
1	1.1	4.1
3	1.3	1.9
10	2.6	2.0
30	0.5	1.0

Our failure to find significant uptake and metabolism of glycolate or phosphoglycolate by randomized cultures of *Chlorella* or *Scenedesmus* is contrary to several earlier reports. Miller et al. (16) observed glycolate disappearance over a period of several hours from *Chlorella* suspensions in the dark and nitrogen atmosphere. Nalewajko, et al. (17) recently reported a small uptake of ¹⁴C-labeled glycolate by a strain of *Chlorella* isolated from the Arctic Ocean. Schou et al. (24) claimed that at pH 2.8 in the light, *Scenedesmus* converted glycolate in a period of 10 minutes into amino acids and sugar phosphates. In particular, glycolate-2-¹⁴C formed P-glycerate which was labeled in carbons 2 and 3. On the basis of that report and the fact that glycolate was formed rapidly by algae during photosynthesis, the assumption was made that the glycolate pathway was present in algae. We have repeated the experiments of Schou et al. and chromatographically analyzed the small amount ¹⁴C products in the cells from the glycolate-2-¹⁴C which was absorbed. Typical results with *Chlorella pyrenoidosa* Chick are shown in table VI and similar results were obtained with *Chlorella pyrenoidosa* (Warburg) and *Scenedesmus obliquus* (Gaffron D-3) which are the same *Scenedesmus* used by Schou et al. (24). The cells were not filtered from the medium as Schou et al. described, so that the percent of glycolate conversion could be estimated. Very little glycolate-¹⁴C metabolism was observed during the 10 minute photosynthesis experiment. Metabolism at pH 6.0 was equal to or

Table VI. Percent Distribution of ^{14}C After Feeding *Chlorella Glycolate-2- ^{14}C*

Approximately 1 μc glycolate-2- ^{14}C was added to suspensions of 2% *Chlorella pyrenoidosa* at pH 2.8 or pH 6.0 in 1 mM phosphate. After 10 min in 3000 ft-c light the suspension was killed with methanol and the % distribution of ^{14}C among the products determined.

Compound	Chick strain		Warburg strain	
	pH 2.8	pH 6.0	pH 2.8	pH 6.0
Glycolate	97.7	98.7	97.7	94.3
Glycine	0.4	0.1	0.3	0.1
Serine	0.6	0.3	0.7	1.0
Other	1.3	1.2	1.4	4.6

greater than that at pH 2.3 for both *Chlorella* and *Scenedesmus*. No estimation of the percent of glycolate metabolized by the *Scenedesmus* was given by Schou, et al., but rather the percent of ^{14}C distributed among glycine, serine, and other labeled products was based on the small amount of glycolate- ^{14}C absorbed. On the basis of the amount of glycolate- ^{14}C used by Schou, et al. and the intensity of their radioautographs, it is likely that we have both observed a limited metabolism of glycolate- ^{14}C by algal suspensions. Several possible reasons might account for the limited (2-3%) conversion of glycolate-2- ^{14}C to other products. An impurity in the glycolate- ^{14}C , such as glyoxylate, might have been present and not detected by the chromatographic procedures employed. The algal cultures were not free of bacteria which could possibly account for some glycolate metabolism. The colorimetric determination of glycolate disappearance might also be criticized, since such a disappearance need not reflect an uptake of the acid by algae. There is, however, the possibility that the random cultures in log phase of growth, as used here, have a very limited ability to metabolize glycolate. Current investigations with synchronized cultures indicate that, at different stages of the life cycle, glycolate may be reabsorbed (E.B. Nelson, N.E. Tolbert, J.L. Hess, unpublished).

Discussion

The wide distribution of glycolate oxidase in higher plants has been well demonstrated. The absence of glycolate oxidase from algae grown in the light on CO_2 is in marked contrast, and as such represents a biochemical difference between the algae and higher plants which should be further investigated in all types of plants.

The assumption that the typical glycolate pathway of higher plant is operating in algae must be modified. The absence of glycolate oxidase, the failure to metabolize much added glycolate- ^{14}C , and the formation of serine from P-glycerate rather than from glycolate are all consistent with the

concept of an incomplete glycolate pathway in algae. Recently other reports have suggested that glycolate oxidase if present in algae may be different from that in higher plants (12, 14, 19). However we wish to emphasize that all the algae tested by us have had an extremely active P-glycolate phosphatase which, like the higher plant enzyme (23), is specific for P-glycolate (K. Swanson, N.E. Tolbert, and J. L. Hess, unpublished). Further the algae synthesize and excrete glycolate during optimum conditions for photosynthesis. Thus the excretion of glycolate by the algae may occur as an end product of carbon metabolism during photosynthesis, because 1 enzyme for further glycolate metabolism is missing.

The absence of glycolate oxidase in algae partially explains our failure to observe any effect of the hydroxymethanesulfonates on glycolate formation by *Chlorella* or *Chlamydomonas* during $^{14}\text{CO}_2$ fixation (32). Addition of the hydroxymethanesulfonates, as inhibitors of glycolate oxidase, to higher plants results in the accumulation of a large percent of the ^{14}C in glycolate (12, 41). The failure of the sulfonate inhibitors to effect glycolate formation in *Chlamydomonas* is consistent with the present report about the absence of glycolate oxidase in algae.

The formation of carboxyl labeled serine and either α -labeled or uniformly labeled glycolate during initial periods of $^{14}\text{CO}_2$ fixation is consistent with the preceding discussion concerning the absence of glycolate oxidase in algae. The labeling pattern in glycolate indicates that it is not the precursor of serine. An alternate pathway, however, for serine formation from 3-P-glycerate, glycerate and hydroxypyruvate could explain the formation of carboxyl labeled serine. Our detection of an active D-glycerate dehydrogenase or glyoxylate reductase in algal extracts would be consistent with this latter pathway. These results are in agreement with a report by Zak that alanine and serine formed by *Chlorella* were carboxyl labeled (39), and the suggestion by Smith et al. (25) that in algae both alanine and serine are formed from P-glycerate. Also, Chang and Tolbert (8) have reported that serine formed by isolated chloroplasts was carboxyl labeled. This pathway for serine formation also explains why serine production in Mn-deficient *Chlorella* does not decrease as glycolate formation decreases.

During glycolate metabolism in higher plants, it is oxidized to glyoxylate and then 2 glyoxylate molecules are converted through glycine to serine and then to glycerate and sugars. The general flow of carbon during photosynthesis may be from glycolate to serine and glycerate in higher plants, but from glycerate to serine in algae. The reasons for the extensive and rapid labeling of glycine and serine by both plants and algae during $^{14}\text{CO}_2$ fixation is not clear. The rate and amount of ^{14}C labeling of serine in brief periods far exceeds that

into other amino acids except carboxyl labeled alanine, aspartate, and glutamate which are formed from the corresponding keto acid.

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