The Pathway of Glycollate Utilization in *Chlorella pyrenoidosa*

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1. Exogenous glycollate was rapidly metabolized in both the light and the dark by photoautotrophically grown *Chlorella pyrenoidosa*. 2. The incorporation of ^{14}C from [$1\text{-}^{14}\text{C}$]glycollate by these cells was inhibited by the tricarboxylic acid-cycle inhibitors monofluoroacetate, diethylmalonate and arsenite, and also by α -hydroxypyrid-2-ylmethanesulphonate and isonicotinylhydrazine. 3. Short-term kinetic experiments showed over 80% of the total ^{14}C present in the soluble fraction from the cells to be in glycine and serine after 10 s. This percentage decreased with time whereas the percentage radioactivity in glycerate increased for up to 30 s then remained steady. The percentage of the total radioactivity present in citrate increased over the experimental period. Malate was the only other tricarboxylic acid-cycle intermediate to become labelled. 4. The kinetic and inhibitor experiments supported the following pathway of glycollate incorporation: glycollate $\rightarrow$ glyoxylate $\rightarrow$ glycine $\rightarrow$ serine $\rightarrow$ hydroxypyruvate $\rightarrow$ glycerate $\rightarrow$ 3-phosphoglycerate $\rightarrow$ 2-phosphoglycerate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate $\rightarrow$ acetyl-CoA. 5. The specific activities of the enzymes catalysing this metabolic sequence in cell-free extracts were great enough to account for the observed rate of glycollate metabolism of 0.25 $\mu\text{mol/h}$ per mg dry wt. of cells in the light.

Glycollate is well established as an early product of photosynthetic carbon dioxide fixation in unicellular algae (Benson & Calvin, 1950; Warburg & Kripphal, 1960; Whittingham & Pritchard, 1963), higher plants (Zelitch, 1959) and isolated chloroplasts (Arnon, Whatley & Allen, 1959). In higher plants glycollate is metabolized to sucrose via glycine and serine (Rabson, Tolbert & Kearney, 1962; Wang & Waygood, 1962; Sinha & Cossins, 1965). The first step in this sequence is catalysed by glycollate oxidase (glycollate-oxygen oxidoreductase, EC 1.1.3.1) and the pathway can be blocked *in vivo* by inhibiting this enzyme, when glycollate accumulates in large amounts (Zelitch, 1957).

Hess & Tolbert (1967) reported the absence of such a pathway from several unicellular green algae and explained the phenomenon of glycollate excretion by algae (Tolbert & Zill, 1956; Whittingham & Pritchard, 1963; Miller, Meyer & Tanner, 1963) as the result of an incomplete glycollate pathway in cells devoid of a higher-plant glycollate oxidase. Recently the generalization that algae excrete, rather than metabolize, glycollate has been questioned by several workers. Zelitch & Day (1968) demonstrated a glycollate-oxidizing enzyme in extracts of air-grown *Chlorella* and *Chlamydomonas*. This enzyme was described as glycollate oxidase,

although oxygen uptake was not measured. Nelson & Tolbert (1969) have reported that the glycollate-oxidizing capacity of a strain of *Chlamydomonas* is regulated by the availability of carbon dioxide during growth. Cells grown on 1% carbon dioxide in air excreted glycollate into the culture medium, whereas cells grown on air did not excrete glycollate. Growth on air de-repressed a glycollate-oxidizing enzyme that differed from the higher-plant enzyme insofar as oxygen could not act as a hydrogen acceptor. 2,6-Dichlorophenol-indophenol was used as the hydrogen acceptor in order to assay the enzyme, which was designated glycollate-2,6-dichlorophenol-indophenol oxidoreductase (Nelson & Tolbert, 1969).

We have shown a strain of *Chlorella pyrenoidosa* to be capable of glycollate oxidation (Lord & Merrett, 1968). This organism is incapable of glycollate excretion even when grown on 5% carbon dioxide in air (Goulding, Lord & Merrett, 1969).

The inability to excrete glycollate did not result from a failure to synthesize glycollate, since excretion could be forced in the presence of α -hydroxypyrid-2-ylmethanesulphonate (Lord & Merrett, 1969), an inhibitor of glycollate oxidase (Zelitch, 1957). The glycollate-oxidizing enzyme of *Chlorella*, like that described by Nelson & Tolbert (1969) for

Chlamydomonas, does not link to oxygen but can be demonstrated by using 2,6-dichlorophenol-indophenol as the electron acceptor (Codd, Lord & Merrett, 1969).

The purpose of the present paper is to describe the pathway of carbon incorporation when exogenous glycollate is metabolized by photoautotrophically grown *Chlorella pyrenoidosa*.

MATERIALS AND METHODS

Growth of organism. *Chlorella pyrenoidosa* (Cambridge Culture Collection, strain 211/8p) was grown photoautotrophically as described by Goulding & Merrett (1966). When carbon-starved cells were required, the culture was aerated in the dark for a further 16 h. Cells were harvested by centrifugation at 500g. For experiments on the incorporation of ^{14}C from $[1\text{-}^{14}\text{C}]\text{glycollate}$, cells were resuspended in 67 mM-potassium phosphate buffer, pH 4.5, at a cell density of 4 mg dry wt./ml. For the preparation of cell-free extracts, cells were washed in distilled water and resuspended in 10 mM-potassium phosphate buffer, pH 7.0, to give approximately a 20–40% (v/v) cell suspension, cell volume being determined by centrifuging at 500g in a graduated centrifuge tube.

Experiments with $[1\text{-}^{14}\text{C}]\text{glycollate}$. Cell samples were obtained at short time-intervals after the addition of $[1\text{-}^{14}\text{C}]\text{glycollate}$ by using an apparatus similar to that of Syrett, Bocks & Merrett (1964). A 20 ml (80 mg dry wt.) portion of a photoautotrophically grown carbon-starved cell suspension was allowed to equilibrate for 30 min at 25°C and light-intensity 12000lx, while being gassed with air at a rate of approx. 4 l/h. At 10 min before zero time 2 μmol of sodium glycollate in 0.1 ml of 67 mM-potassium phosphate buffer, pH 4.5, was added. At zero time, 2 μmol of sodium $[1\text{-}^{14}\text{C}]\text{glycollate}$ containing 30 μCi in 1.0 ml of 67 mM-potassium phosphate buffer, pH 4.5, was added and 2 ml samples were removed at short time-intervals into 8 ml of ethanol precooled to -40°C. Products of $[1\text{-}^{14}\text{C}]\text{glycollate}$ metabolism were separated by two-dimensional paper chromatography and identified as described previously (Merrett & Goulding, 1967).

The incorporation of ^{14}C from $[1\text{-}^{14}\text{C}]\text{glycollate}$ into various cell fractions and the release of $^{14}\text{CO}_2$ were determined as described previously for $[^{14}\text{C}]\text{acetate}$ (Goulding & Merrett, 1966).

Preparation of cell extracts. Suspensions of freshly harvested cells were disrupted by passage through an ice-cold French pressure cell at 20000 lb/in² (Milner, Lawrence & French, 1950). After centrifugation at 500g at 0–4°C for 5 min to remove intact cells and cell debris, the supernatant was centrifuged at 10000g for 10 min to remove larger particulate matter and was then used immediately in enzyme assays.

Protein determination. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) by using a standard curve prepared for crystalline bovine serum albumin.

Enzyme assays. All assays were performed on extracts of photoautotrophically grown *Chlorella* cells. All assays were carried out in silica cuvettes (3.0 ml volume; 1 cm light-path) with a Unicam SP.500 spectrophotometer.

Glycollate-2,6-dichlorophenol-indophenol oxidoreductase. This enzyme was assayed anaerobically by measuring 2,6-dichlorophenol-indophenol reduction (Zelitch & Day, 1968). The following additions were made to a 3.0 ml Thunberg cuvette (1 cm light-path): 200 μmol of sodium pyrophosphate buffer, pH 8.7, containing 0.3 μmol of 2,6-dichlorophenol-indophenol, 0.2 ml of enzyme (containing 3.6 mg of protein) and water to give a final volume of 3.0 ml with 12.5 μmol of sodium glycollate in the side arm. The cuvette was evacuated and flushed ten times with O_2 -free N_2 and dye reduction was measured at 600 nm after the glycollate had been tipped in. No dye reduction occurred in a control cuvette containing all components except glycollate. Under the conditions of the assay a decrease in extinction of 0.01 corresponded to the oxidation of 1.8 nmol of glycollate.

Phosphoglycollate phosphatase (EC 3.1.3.18). This enzyme was assayed by measuring P_i released from phosphoglycollate by the method of Anderson & Tolbert (1966). The reaction mixture contained, in a final volume of 3.0 ml, 100 μmol of sodium cacodylate buffer, pH 6.3, 10 μmol of phosphoglycollate and 6 μmol of MgSO_4 . After the addition of cell extract (containing 1.8 mg of protein) the mixture was incubated at 30°C for 10 min.

The reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid and, after the removal of denatured protein by centrifugation, the P_i present in a portion of the supernatant was determined by the method of King (1932). Two controls, one with enzyme omitted and one with phosphoglycollate omitted, were run to correct for P_i that was liberated other than by the action of phosphoglycollate phosphatase.

Glyoxylate reductase (EC 1.1.1.26). This enzyme was assayed spectrophotometrically by measuring a decrease in extinction at 340 nm consequent on the oxidation of NADH by glyoxylate (Zelitch, 1955). The reaction mixture contained, in a final volume of 3.0 ml, 100 μmol of potassium phosphate buffer, pH 6.5, 1 μmol of NADH and cell extract (containing 0.33 mg of protein). The reaction was started by the addition of 50 μmol of glyoxylate.

No NADH was oxidized in a control cuvette containing all components except glyoxylate. Assuming ϵ_{340} of NADH to be 6.2×10^3 , the specific activity of the enzyme was calculated as μmol of NADH oxidized/h per mg of protein.

Glyoxylate-glutamate aminotransferase (EC 2.6.1.4). This enzyme was assayed by measuring the formation of $[^{14}\text{C}]\text{glycine}$ from $[1,2\text{-}^{14}\text{C}]\text{glyoxylate}$. The assay mixture, composed of 20 μmol of $[1,2\text{-}^{14}\text{C}]\text{glyoxylate}$ containing 1 μCi (25 μmol) of DL-glutamic acid, 0.1 μmol of pyridoxal phosphate, 200 μmol of potassium phosphate buffer, pH 7.5, and cell extract (containing 1.5 mg of protein) in a final volume of 2.0 ml was incubated at 30°C for 30 min. The reaction was stopped by heating on a boiling-water bath for 5 min, and unchanged $[^{14}\text{C}]\text{glyoxylate}$ was removed by passage down a column (1 cm $\times$ 6 cm) of Dowex 1 (Cl^- form), which was then washed with 2 ml of water. The combined effluents were evaporated to a small volume on a rotary film evaporator at 45°C under reduced pressure and subjected to two-dimensional co-chromatography on paper with 10 μg of carrier glycine with the solvent systems of Wilson & Calvin (1955). The glycine spot was located by spraying

with ninhydrin and its radioactivity was determined with a Nuclear-Chicago Actigraph II gas-flow chromatogram scanner.

Serine hydroxymethyltransferase (EC 2.1.2.1). This enzyme was assayed by the method of Taylor & Weissbach (1965), which measures the liberation of [^{14}C] formaldehyde from [$3\text{-}^{14}\text{C}$]serine. A reaction mixture containing $3\mu\text{mol}$ of [$3\text{-}^{14}\text{C}$]serine containing $1\mu\text{Ci}$ ($0.3\mu\text{mol}$) of pyridoxal phosphate, $2.5\mu\text{mol}$ of tetrahydrofolate, $12\mu\text{mol}$ of 2-mercaptoethanol, $200\mu\text{mol}$ of potassium phosphate buffer, pH 7.4, and enzyme (containing 0.5–2.5 mg of protein) in 1.75 ml was incubated at 30°C for 30 min and then treated as described by Taylor & Weissbach (1965).

Hydroxypyruvate reductase (EC 1.1.1.29). This enzyme was determined by measuring the decrease in extinction at 340 nm consequent on the oxidation of NADH by hydroxypyruvate. The reaction mixture contained, in a final volume of 3.0 ml, $100\mu\text{mol}$ of potassium phosphate buffer, pH 6.3, $2\mu\text{mol}$ of NADH and cell extract (containing 0.175 mg of protein). The reaction was started by the addition of $50\mu\text{mol}$ of hydroxypyruvate. No NADH was oxidized in a control cuvette containing all components except hydroxypyruvate.

D-Glycerate dehydrogenase (EC 1.1.1.29). The enzyme catalysing the reverse reaction of the hydroxypyruvate reductase was assayed by measuring an increase in extinction at 340 nm consequent on the reduction of NAD^+ by D-glycerate. The reaction mixture contained, in a final volume of 3.0 ml, $100\mu\text{mol}$ of potassium phosphate buffer, pH 6.5, $2\mu\text{mol}$ of NAD^+ and cell extract (containing 0.33 mg of protein).

The reaction was started by the addition of $50\mu\text{mol}$ of D-glycerate. No NAD^+ was reduced in a control cuvette containing all components except D-glycerate.

Conversion of serine into glycerate. This conversion was demonstrated by incubating serine and pyruvate with enzyme in the presence of pyridoxal phosphate and NADH. The hydroxypyruvate formed from serine by transamination with pyruvate was reduced to glycerate with the concomitant oxidation of NADH, which was followed by measuring a decrease in extinction at 340 nm. The reaction mixture contained, in a final volume of 3.0 ml, $200\mu\text{mol}$ of potassium phosphate buffer, pH 8.3, $20\mu\text{mol}$ of pyruvate, $0.1\mu\text{mol}$ of pyridoxal phosphate, $1\mu\text{mol}$ of NADH and cell extract (containing 0.24 mg of protein). The reaction was started by the addition of $20\mu\text{mol}$ of serine. Preliminary assay showed that the cell extract contained negligible lactate dehydrogenase activity. No NADH oxidation occurred in control cuvettes from which either pyruvate or serine had been omitted.

Malate dehydrogenase (EC 1.1.1.37). This enzyme was assayed by measuring the decrease in extinction at 340 nm consequent on the oxidation of NADH by oxaloacetate. The reaction mixture contained, in a final volume of 3.0 ml, $100\mu\text{mol}$ of potassium phosphate buffer, pH 6.5, $1\mu\text{mol}$ of NADH and cell extract (containing 0.33 mg of protein). The reaction was started by the addition of $50\mu\text{mol}$ of oxaloacetate. No NADH was oxidized in a control cuvette containing all components except oxaloacetate.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31). This enzyme catalyses the formation of oxaloacetate from

phosphoenolpyruvate and CO_2 . The formation of oxaloacetate can be demonstrated by its reduction to malate by cell extracts shown to contain excess of malate dehydrogenase.

The decrease in extinction at 340 nm consequent on the oxidation of NADH by oxaloacetate was a measure of the phosphoenolpyruvate carboxylase activity. The reaction mixture contained, in a final volume of 3.0 ml, $100\mu\text{mol}$ of tris-HCl buffer, pH 8.2, $5\mu\text{mol}$ of NaHCO_3 , $10\mu\text{mol}$ of MgCl_2 , $10\mu\text{mol}$ of 2-mercaptoethanol, $1\mu\text{mol}$ of NADH and cell extract (containing 0.6 mg of protein). The reaction was started by the addition of $5\mu\text{mol}$ of phosphoenolpyruvate. No significant NADH oxidation occurred in a control cuvette containing all components except phosphoenolpyruvate.

Glyoxylate carboligase. This enzyme (Krakow & Barkulis, 1956) was assayed manometrically by measuring the rate of anaerobic evolution of CO_2 from glyoxylate. Double-armed Warburg flasks contained $80\mu\text{mol}$ of potassium phosphate buffer, pH 6.4, $0.5\mu\text{mol}$ of thiamin pyrophosphate, $10\mu\text{mol}$ of MgCl_2 and cell extract (containing 1.5 mg of protein) and water to a total volume of 2.2 ml in the main compartment. The flasks were gassed with O_2 -free N_2 . After incubation at 30°C , $10\mu\text{mol}$ of glyoxylate was added from one side arm. The reaction was allowed to proceed for 10, 20 or 30 min, and was then stopped by the addition of 0.2 ml of 1 M H_2SO_4 from the other side arm.

Glyoxylate-cycle enzymes. Isocitrate lyase (EC 4.1.3.1) and malate synthetase (EC 4.1.3.2) were assayed as described previously (Goulding & Merrett, 1966).

Radioactive materials. All radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Sodium [$1\text{-}^{14}\text{C}$]glycollate was dissolved in the minimum quantity of water and purified before use by paper chromatography on Whatman no. 3 paper in ethanol-aq. ammonia (sp.gr. 0.88)–water (20:1:4, by vol.) (Long, Quayle & Stedman, 1951).

Other materials. Nicotinamide nucleotides, pyridoxal phosphate, tetrahydrofolate and phosphoenolpyruvate were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., phosphoglycollate was purchased from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A., as the tricyclohexylammonium salt and used in this form, α -hydroxypyrid-2-ylmethanesulphonate was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., 3-(3,4-dichlorophenyl)-1,1-dimethylurea was from K & K Laboratories, Plainview, N.Y., U.S.A., and hydroxypyruvate was from Calbiochem, Los Angeles, Calif., U.S.A. All other materials were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest purity commercially available.

RESULTS

Incorporation of ^{14}C from [$1\text{-}^{14}\text{C}$]glycollate into Chlorella cells. Photoautotrophically grown *Chlorella* cells rapidly incorporated ^{14}C from [$1\text{-}^{14}\text{C}$]glycollate into various cell fractions in the light and the dark, showing that exogenous glycollate was rapidly transformed into a wide range of cell constituents. This incorporation was inhibited by

Table 1. *Effects of α -hydroxypyrid-2-ylmethanesulphonate and isonicotinylhydrazine on the photoassimilation of $[1-^{14}\text{C}]$ glycollate by *Chlorella pyrenoidosa* in the light and in the dark*

A 2ml sample containing 8mg dry wt. of autotrophically grown carbon-starved *Chlorella* in 67mm-potassium phosphate buffer, pH 4.5, in a Warburg flask was allowed to equilibrate in the light or in the dark and in the presence or absence of inhibitors for 30min. Then 10 μmol of $[1-^{14}\text{C}]$ glycollate containing 2 μCi was added from the side arm. The cell suspension was sampled after 30min. The light-intensity was 12000lx and the temperature 25°C. The results given represent total nCi of ^{14}C incorporated (means of duplicate flasks) per fraction. Abbreviation: α -HPMS, α -hydroxypyrid-2-ylmethanesulphonate.

Fraction	Control		10mm- α -HPMS		10mm-Isonicotinylhydrazine	
	Light	Dark	Light	Dark	Light	Dark
Water extract	95.2	61.8	48.7	50.1	90.2	130.0
Ethanol extract	34.0	3.6	2.1	1.0	5.6	1.5
Acid hydrolysate	58.0	16.0	1.6	1.3	7.8	2.0
Cell residue	14.8	4.1	1.0	0.3	4.7	0.9
Total assimilated	202.0	85.5	53.4	52.7	108.3	134.4

Table 2. *Effect of sodium glycollate on photosynthetic $^{14}\text{CO}_2$ fixation by *Chlorella pyrenoidosa**

A 2ml sample containing 8mg dry wt. of autotrophically grown carbon-starved *Chlorella* cells suspended in 67mm-potassium phosphate buffer, pH 6.5, in a Warburg flask was allowed to equilibrate in the light in the presence or absence of 10mm-sodium glycollate for 30min. Then 20 μmol of $\text{NaH}^{14}\text{CO}_3$ containing 2 μCi was added from the side arm. The cell suspension was sampled after 30min. The light-intensity was 12000lx and the temperature 25°C. Figures represent total nCi of ^{14}C incorporated per fraction (means of duplicate flasks).

Fraction	Control	10 mm-Glycollate
Supernatant	21.6	18.1
Water extract	212.0	238.0
Ethanol extract	285.0	308.0
Acid hydrolysate	342.0	315.0
Cell residue	66.5	66.0
Total assimilated	927.1	945.1

10mm- α -hydroxypyrid-2-ylmethanesulphonate, an inhibitor of glycollate oxidase (Zelitch, 1957), and by 10mm-isonicotinylhydrazine, which inhibits the conversion of glycine into serine (Table 1).

This result indicated that glycollate was being further metabolized via glyoxylate and glycine. It was unlikely that glycollate was being completely oxidized via glyoxylate and formate to $^{14}\text{CO}_2$ which was then being re-fixed by the photosynthetic carbon reduction cycle, because it has been shown that 10mm-isonicotinylhydrazine does not inhibit total $^{14}\text{CO}_2$ fixation by *Chlorella* (Whittingham & Pritchard, 1963). Evidence in support of this view was the observation that unlabelled glycollate did not affect the rate of $^{14}\text{CO}_2$ fixation in *Chlorella* (Table 2).

In the presence of α -hydroxypyrid-2-ylmethanesulphonate and isonicotinylhydrazine, most of the total ^{14}C incorporated by the cells was shown to be present in the water extract (Table 1) and it was considered likely that the bulk of this represented unchanged $[1-^{14}\text{C}]$ glycollate that had entered the cells.

The effect of various metabolic inhibitors on $[1-^{14}\text{C}]$ glycollate incorporation into various cell fractions of *Chlorella* and the release of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glycollate is shown in Table 3.

Kinetics of $[1-^{14}\text{C}]$ glycollate assimilation. The early products of $[1-^{14}\text{C}]$ glycollate incorporation were determined by feeding the cells on unlabelled glycollate and when this was nearly metabolized supplying a pulse of $[1-^{14}\text{C}]$ glycollate and taking cell samples. Label from $[1-^{14}\text{C}]$ glycollate rapidly appeared in glycine and serine, and in glycerate. At the earliest time (10s) 80% of the radioactivity on the chromatogram was located in glycine and serine and this percentage gradually fell with time, whereas that in citrate and malate increased (Fig. 1).

The presence of 10mm- α -hydroxypyrid-2-ylmethanesulphonate resulted in a 90% decrease of the total ^{14}C present in the soluble fraction of the cells, the only labelled compounds present being glycine, serine and glycerate.

*Enzymic reactions in *Chlorella* extracts*

Glycollate oxidation. Cell-free extracts were capable of oxidizing glycollate and this reaction was demonstrated by using 2,6-dichlorophenol-indophenol as the hydrogen acceptor and measuring dye reduction. The product of glycollate oxidation by *Chlorella* extracts forms a phenylhydrazone derivative that absorbs at 324nm, indicating that the product is glyoxylate (Lord & Merrett, 1968).

Table 3. *Effects of various inhibitors on the photoassimilation of [1-¹⁴C]glycollate and the evolution of ¹⁴CO₂ from [1-¹⁴C]glycollate by Chlorella pyrenoidosa in the light*

Control values are total nCi of ¹⁴C incorporated into each fraction or liberated per flask; all other results are expressed as percentages of the control (means of duplicate flasks). Abbreviations: α -HPMS, α -hydroxypyrid-2-ylmethanesulphonate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Fraction	Control	10 mM- α -HPMS	10 mM- Isonico- tynyl- hydrazine	4 mM- Mono- fluoro- acetate	10 mM- Diethyl malonate	10 mM- Sodium hypo- phosphite	4 mM- Sodium arsenite	1 μ M- DCMU
Water extract	95.2	51.2	94.7	93.0	51.6	99.8	80.0	64.4
Ethanol extract	34.0	6.2	16.5	4.5	41.8	95.0	51.5	19.4
Acid hydrolysate	58.0	2.8	13.4	2.7	50.0	104.0	41.6	37.0
Cell residue	14.8	6.7	31.8	2.2	51.5	111.7	63.0	63.0
Total assimilated	202.0	26.4	53.6	52.8	50.1	101.7	65.8	52.3
¹⁴ CO ₂ evolved	83.8	4.2	26.8	8.3	21.8	—	36.6	137.0

Fig. 1. Variation with time of the percentage distribution of ¹⁴C from [1-¹⁴C]glycollate incorporated by *Chlorella pyrenoidosa* into glycine and serine (○), glycerate (●), citrate (■) and malate (□). For experimental procedure see the Materials and Methods section.

The *Chlorella* enzyme does not link to oxygen and is therefore different from the higher-plant flavo-protein glycollate oxidase (EC 1.1.3.1).

Glycine formation. *Chlorella* extracts transaminated [1,2-¹⁴C]glyoxylate to [¹⁴C]glycine in the presence of DL-glutamate (Table 4). Glycine was the only radioactive amino acid formed; no serine was detected.

The [¹⁴C]glycine was located on paper chromato-

grams by spraying with ninhydrin (see the Materials and Methods section) and although this procedure may result in the liberation of some ¹⁴CO₂ from glycine, the enzyme activity as determined by amount of [¹⁴C]glycine remaining on the chromatogram was still sufficiently high to account for the observed rate of glycollate metabolism by photoautotrophically grown cells.

The results shown in Table 4 indicate that pyridoxal phosphate is necessary for maximum enzyme activity, and the high value of the control with boiled enzyme suggests that some non-enzymic transamination of glyoxylate, as observed by Nakada & Weinhouse (1953) and Fleming & Crosbie (1960), may have occurred.

Glycine-serine interconversion. Cell-free extracts of *Chlorella* contained serine hydroxymethyltransferase (Table 5). The formation of the radioactive C₁ unit from [3-¹⁴C]serine showed an absolute requirement for enzyme and tetrahydrofolate, and pyridoxal phosphate was necessary for maximum activity. The conversion of glycine into serine would require a C₁ unit, which we assume arises from the enzymic splitting of glycine into such a unit, that would then condense with another molecule of glycine to form serine.

Conversion of serine into glycerate. Serine was converted into glycerate by *Chlorella* extracts. The addition of pyruvate to a reaction mixture containing enzyme, serine and NADH facilitated the deamination of serine to hydroxypyruvate and its reduction to glycerate. This oxidation of NADH in the presence of pyruvate was not due to lactate dehydrogenase activity, since no oxidation of NADH occurred in the presence of either pyruvate or serine singly, but only when both were present.

The reduction of hydroxypyruvate to glycerate by hydroxypyruvate reductase and the reverse oxidation of D-glycerate to hydroxypyruvate by D-glycerate dehydrogenase were also demonstrated.

Table 4. *Formation of [¹⁴C]glycine from [1,2-¹⁴C]glyoxylate by Chlorella extracts*

For details of experimental procedure see the Materials and Methods section.

Assay conditions	¹⁴ C in glycine (c.p.m.)	Relative rate (%)
No amino donor	5340	(100)
DL-Glutamate	30890	580
Boiled enzyme + DL-glutamate	13870	260
Minus pyridoxal phosphate + DL-glutamate	21470	403

Table 5. *Serine hydroxymethyltransferase activity in Chlorella extracts*

For details of assay procedure see the Materials and Methods section.

Assay system	¹⁴ C released from [3- ¹⁴ C]serine (d.p.m.)
Complete system + 0.05 ml of enzyme	70560
Complete system + 0.15 ml of enzyme	215250
Complete system + 0.25 ml of enzyme	294000
Minus enzyme	11690
Minus tetrahydrofolate (0.25 ml of enzyme)	11200
Minus pyridoxal phosphate (0.25 ml of enzyme)	101150

Additional reactions of glyoxylate. *Chlorella* extracts were shown to convert glyoxylate, the product of glycollate oxidation, into glycine, but because of the wide variety of metabolic reactions involving glyoxylate, other enzymic reactions of which glyoxylate is the substrate were also investigated. *Chlorella* extracts contained an active NADH-linked glyoxylate reductase.

During the growth of some bacteria on glycollate (for example, see Kornberg & Gotto, 1961), two molecules of glyoxylate, resulting from glycollate oxidation, are converted into one molecule of tartronic semialdehyde and carbon dioxide, a reaction catalysed by the enzyme glyoxylate carboligase (Krakow & Barkulis, 1956). We were unable to demonstrate any glyoxylate carboligase activity in *Chlorella* extracts.

The glyoxylate-cycle enzymes isocitrate lyase and malate synthetase were detectable in photoautotrophically grown *Chlorella* extracts, but the activity was very low for each enzyme, as demonstrated previously (Syrett, Merrett & Bocks, 1963).

Phosphoenolpyruvate carboxylase. *Chlorella* extracts were shown to contain an active malate dehydrogenase. Therefore it was possible to assay phosphoenolpyruvate carboxylase spectrophotometrically in such extracts by reducing any oxaloacetate formed as a result of the carboxylation reaction to malate in the presence of NADH. Little reaction occurred in the absence of phosphoenolpyruvate, but a substantial oxidation of NADH occurred in the presence of phosphoenolpyruvate without the addition of bicarbonate, probably because of CO₂ dissolved in the buffer at the pH of the assay (8.2). However, maximum NADH oxidation occurred in the presence of added phosphoenolpyruvate and bicarbonate. Addition of catalytic amounts of acetyl-CoA, shown by Canovas & Kornberg (1966) greatly to stimulate the rate of oxaloacetate formation by the enzyme in extracts of glycerol-grown *Escherichia coli*, had no effect on the rate of reaction in *Chlorella* extracts.

metrically in such extracts by reducing any oxaloacetate formed as a result of the carboxylation reaction to malate in the presence of NADH. Little reaction occurred in the absence of phosphoenolpyruvate, but a substantial oxidation of NADH occurred in the presence of phosphoenolpyruvate without the addition of bicarbonate, probably because of CO₂ dissolved in the buffer at the pH of the assay (8.2). However, maximum NADH oxidation occurred in the presence of added phosphoenolpyruvate and bicarbonate. Addition of catalytic amounts of acetyl-CoA, shown by Canovas & Kornberg (1966) greatly to stimulate the rate of oxaloacetate formation by the enzyme in extracts of glycerol-grown *Escherichia coli*, had no effect on the rate of reaction in *Chlorella* extracts.

DISCUSSION

It has been suggested (Hess & Tolbert, 1967) that unicellular green algae are incapable of glycollate metabolism and excrete glycollate formed during photosynthesis into the suspending medium. The strain of *Chlorella* investigated in this work fails to excrete glycollate under conditions favouring glycollate formation (Goulding *et al.* 1969). Further, it has been shown in the present paper that the organism can also rapidly metabolize exogenous glycollate and that photoautotrophically grown cells are capable of incorporating ¹⁴C from [1-¹⁴C]-glycollate into various cell fractions (Table 1).

The effect of certain metabolic inhibitors on [1-¹⁴C]glycollate assimilation and ¹⁴CO₂ release from [1-¹⁴C]glycollate (Tables 1 and 3) gives an indication of the pathway of glycollate metabolism. α -Hydroxypyrid-2-ylmethanesulphonate inhibits glycollate metabolism by photoautotrophically grown cells, indicating that the first step in glycollate metabolism is its oxidation to glyoxylate. The glyoxylate is not oxidized to CO₂ via formate as we were unable to demonstrate an NAD- or NADP-linked formate dehydrogenase in *Chlorella*, and in confirmation of this, sodium hypophosphite, an inhibitor of formate dehydrogenase (Campbell, 1955), has no effect on glycollate metabolism (Table 3).

These observations were supported by the fact that sugar phosphates and related compounds, known to be primary products of photosynthetic CO₂ fixation, were not formed as early products of [1-¹⁴C]glycollate assimilation (Fig. 1). The failure of unlabelled glycollate to decrease ¹⁴CO₂ fixation (Table 2) is additional evidence against the complete oxidation of glycollate and re-fixation of the CO₂ released. Isonicotinylhydrazine, which inhibits the conversion of glycine into serine (Whittingham & Pritchard, 1963), also inhibits

Table 6. *Specific activities of enzymes demonstrated in Chlorella extracts*

Enzyme	Specific activity (μmol of substrate transformed/h per mg of protein)
Phosphoglycollate phosphatase	0.20
Glycollate-2,6-dichlorophenol- indophenol oxidoreductase	0.24
Glyoxylate reductase	4.14
Glyoxylate-glutamate amino- transferase	4.00
Glyoxylate carboligase	Not detected
Serine hydroxymethyltransferase	0.38
Conversion of serine into glycerate	3.30
Hydroxypyruvate reductase	17.00
D-Glycerate dehydrogenase	27.00
Malate dehydrogenase	13.20
Phosphoenolpyruvate carboxylase	0.44
Isocitrate lyase	0.02
Malate synthetase	0.02

glycollate metabolism. This suggests the conversion of glyoxylate into glycine, which is further metabolized to a C_3 compound, serine. We were unable to detect glyoxylate carboligase activity in *Chlorella* extracts, so glyoxylate was not being converted into an alternative C_3 compound, tartronic semi-aldehyde, a reaction shown to occur during the growth of some bacteria on glycollate (Kornberg & Gotto, 1961).

The tricarboxylic acid-cycle inhibitors monofluoroacetate, diethylmalonate and sodium arsenite inhibit the incorporation of glycollate carbon by the cells and the release of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glyoxylate (Table 3), indicating that the tricarboxylic acid cycle plays an important role in the route of glycollate metabolism. Monofluoroacetate also severely inhibited incorporation of glycollate carbon into the ethanol-soluble lipid fraction of the cells, and may have been acting on the enzyme catalysing the conversion of acetyl-CoA into malonyl-CoA. On the basis of these observations a scheme of reactions is proposed to account for the metabolism of glycollate by *Chlorella* (Scheme 1). An identical scheme has been postulated by Shah & Rogers (1969) to account for acetyl-CoA formation from glycollate in maize chloroplasts in their study of chloroplast terpenoid biosynthesis. However, these workers did not present any enzymic evidence for the proposed pathway.

The postulated pathway accounts for the distribution of label from $[1-^{14}\text{C}]$ glycollate in the soluble fraction of the cells (Fig. 1). $[1-^{14}\text{C}]$ Glycollate would be metabolized via a series of intermediates each labelled at C-1 to $1-^{14}\text{C}$ -labelled phosphoenolpyruvate. Acetyl-CoA derived from

phosphoenol $[1-^{14}\text{C}]$ pyruvate via pyruvate and pyruvate oxidase would be unlabelled, whereas oxaloacetate formed by the carboxylation of phosphoenol $[1-^{14}\text{C}]$ pyruvate would be labelled and would give rise to citrate labelled in only one carbon atom. The citrate would then be metabolized via the tricarboxylic acid-cycle reactions and the labelled carbon atom would be lost at the first decarboxylation stage of the cycle resulting in unlabelled α -oxoglutarate. Glutamate and tricarboxylic acid-cycle intermediates subsequently formed would be unlabelled. Although it is possible that some randomization of label between C-1 and C-4 of oxaloacetate may have occurred from partial reversal of the reactions to fumarate, the results we obtained did not suggest this had occurred. The only labelled intermediates of the postulated sequence that could be expected to be present on chromatograms run in the solvent systems used in this work would be glycine, serine, glycerate, phosphoglycerate, phosphoenolpyruvate, citrate and possibly malate, all of which, except phosphoenolpyruvate and phosphoglycerate, were present on the experimental chromatograms.

Enzyme studies also provide convincing evidence for this pathway of glycollate metabolism. The oxidation of glycollate to glyoxylate, the conversion of glyoxylate into glycine, serine hydroxymethyltransferase activity and the conversion of serine into glycerate via hydroxypyruvate have all been demonstrated in *Chlorella* extracts (Table 6). Since the cell-free extracts also contain glycerate kinase (J. M. Lord & M. J. Merrett, unpublished work) glycerate is probably metabolized via the well-established reactions of the Embden-Meyerhof-Parnas pathway to pyruvate, which is converted into acetyl-CoA and enters the tricarboxylic acid cycle.

From the data in Table 1 it was calculated that the rate of glycollate uptake by photoautotrophically grown *Chlorella* was $0.25 \mu\text{mol/h}$ per mg dry wt. of cells in the light and $0.11 \mu\text{mol/h}$ per mg dry wt. of cells in the dark. The specific activities of the various enzymes catalysing the component reactions of the proposed metabolic route are sufficient to account for the overall rate of glycollate metabolism (Table 6).

Although the present experiments were carried out with photoautotrophically grown cells, we have shown that glycollate will support photoheterotrophic growth of this organism (J. M. Lord & M. J. Merrett, unpublished work). It is probable that the same metabolic route would occur during growth on glycollate, but during this growth intermediates of the tricarboxylic acid cycle will be required as precursors for the synthesis of cell constituents. It was considered possible that malate synthetase may play an anaplerotic role

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