Biochem. J. (1975) **148**, 321–328
Printed in Great Britain

The Localization of Glycollate-Pathway Enzymes in *Euglena*

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(*Received* 15 *May* 1974)

1. Isolation of organelles from broken-cell suspensions of phototrophically grown *Euglena gracilis* Klebs was achieved by isopycnic centrifugation on sucrose gradients. 2. Equilibrium densities of 1.25g/cm^3 for peroxisome-like particles, 1.22g/cm^3 for mitochondria and 1.17g/cm^3 for chloroplasts were recorded. 3. The enzymes glycollate dehydrogenase, glutamate–glyoxylate aminotransferase, serine–glyoxylate aminotransferase, aspartate–α-oxoglutarate aminotransferase, hydroxypyruvate reductase and malate dehydrogenase were present in peroxisome-like particles. 4. Unlike higher plants glycollate dehydrogenase and glutamate–glyoxylate aminotransferase were present in the mitochondria of *Euglena*. 5. Rates of glycollate and D-lactate oxidation were additive in the mitochondria, and, although glycollate dehydrogenase was inhibited by cyanide, D-lactate dehydrogenase activity was unaffected. 6. Glycollate oxidation was linked to O_2 uptake in mitochondria but not in peroxisome-like particles. This glycollate-dependent O_2 uptake was inhibited by antimycin A or cyanide. 7. The physiological significance of glycollate metabolism in *Euglena* mitochondria is discussed, with special reference to its role in photorespiration in algae.

The presence of the glycollate pathway of metabolism is well-documented in unicellular green algae (Lord & Merrett, 1970; Bruin *et al.*, 1970). Glycollate formed from CO_2 during photosynthesis is oxidized to glyoxylate and subsequently converted via glycine and serine into glycerate. Studies on the intracellular localization of the glycollate-pathway enzymes in higher plants have established their presence in leaf peroxisomes (Tolbert *et al.*, 1968). These organelles of the type morphologically described as microbodies (Hruban & Rechcigl, 1969) are characterized by a single limiting membrane and a granular matrix (Frederick & Newcomb, 1969). Enzymically these organelles are characterized by the association of one or more flavin oxidase enzymes with an excess of catalase and the presence of several enzymes of the glycollate pathway including glutamate–glyoxylate aminotransferase and NADH hydroxypyruvate reductase (Yamazaki & Tolbert, 1969; Rehfeld & Tolbert, 1972).

The oxidation of glycollate by the FMN flavoprotein glycollate oxidase of higher plants results in the production of H_2O_2 which is subsequently reduced by the catalase present in the peroxisomes. The algal glycollate dehydrogenase differs in that O_2 is not the immediate acceptor for glycollate electrons (Codd *et al.*, 1969; Nelson & Tolbert, 1970) so that the algal enzyme need not of necessity be associated with catalase in an organelle of the peroxisome type. Even so, electron microphotographs have revealed organelles of similar morphology to microbodies in *Chlorella* (Gergis, 1971; Pulich & Ward, 1973) and *Euglena* (Graves *et al.*, 1971; Brody & White, 1972) and although Lord & Merrett (1971) reported that glycollate dehydrogenase was a particulate enzyme in *Euglena* the complete intracellular localization of glycollate pathway enzymes in algae has not been reported. In view of this and the failure of Bégin-Heick (1973) to isolate distinct microbody and mitochondrial fractions from acetate-grown *Euglena*, we have further investigated the intracellular localization of glycollate-pathway enzymes in *Euglena*.

Materials and Methods

Growth of alga

Asynchronous cultures of *Euglena gracilis* Klebs strain Z were grown at 25°C in the phototrophic growth medium of Cramer & Myers (1952) at a continuous light intensity of 6000lx provided by banks of fluorescent tubes (Osram white). The initial inoculum added to 6 litres of medium was 150ml of an exponential-phase culture containing approx. 10^5cells/ml. Cultures, after 48h of growth on 5% CO_2 in air followed by 24h growth on air, were harvested in early exponential growth.

Preparation of cell extracts

Cells were harvested by centrifugation at 500*g* for 5min, washed once with, and then resuspended in, 0.4M-sucrose in 1mM-EDTA, pH7.5, to give a 50% (v/v) cell suspension, cell volume being determined

by centrifuging at 500*g* for 5min in a graduated centrifuge tube. The cell suspension was added to an equal volume of no. 7 glass Ballotini beads in a mortar and gently ground in the cold for 5min. The beads were washed three times with 0.5vol. of buffer, the combined washings centrifuged at 250*g* for 5min, and the supernatant was decanted. The pellet was resuspended, centrifuged at 250*g* for 5min, and the supernatants were combined. After centrifuging at 250*g* for 5min to remove any remaining cell debris, the crude extract was used for the isolation or organelles. The method of disruption gave a marginally better yield of organelles than in previous experiments, when cells were shaken with ballotini in a Braun MSK rotary cell homogenizer (Davis & Merrett, 1973). All sucrose solutions were prepared in 20mM-glycylglycine buffer, pH7.5.

Separation of Euglena organelles by sucrose-density-gradient centrifugation

A continuous sucrose gradient was formed by using two interconnected vessels with an outlet from the second vessel connected to a peristaltic pump. To the first vessel was added 17.5ml of 0.5M sucrose in 1mM-EDTA, pH7.5, and 15.0ml of 2.5M-sucrose in 1mM-EDTA was added to the second vessel. The clip between the two vessels was opened and sucrose solution from the second vessel was slowly withdrawn by means of the peristaltic pump into a centrifuge tube containing in the bottom a layer of 3ml of 2.5M-sucrose in 1mM-EDTA, pH7.5. The efficient mixing of the two sucrose solutions in the second vessel was ensured by rapid bubbling with air. The linearity of the gradient was established with an Abbe refractometer to determine the density of 1ml fractions removed from the gradient.

The crude cell extract (5ml) was layered on top of the sucrose gradient; the centrifuge tube was then carefully placed in the bucket of a 3×40ml swing-out rotor and spun at 20000rev./min (65000$g_{av.}$) for 4½h at 0–4°C in an MSE Superspeed 40 centrifuge. At the end of the run the tube was removed, clamped in a tube piercer and 1ml fractions for enzyme assay were removed through a needle from the bottom of the tube.

Differential centrifugation of Euglena extracts

Mitochondria were obtained in bulk by differential centrifugation of *Euglena* extracts prepared by suspending cells in 0.4M-sucrose solution containing 1% (w/v) bovine serum albumin and grinding as before. The extract was centrifuged at 250*g* for 5min to remove cell debris, then at 3000*g* for 2min to spin down chloroplasts and finally at 10000*g* for 30min to spin down mitochondria. The mitochondria were resuspended in 25mM-Hepes buffer,* pH7.0, containing 0.3M-sorbitol, 0.5mM-EDTA and 1% (w/v) bovine serum albumin.

Oxygen uptake

O_2 uptake by mitochondrial preparations was measured by using a Rank oxygen electrode in a continuously stirred perspex vessel round which was circulated water from a thermostatically controlled water bath at 25°C. The vessel contained 0.4ml of mitochondrial preparation and 0.4ml of the suspending medium; 0.2ml of sodium glycollate solution, pH7.0, (final concentration 40mM) was added via the central capillary through the cylindrical plunger used to close the vessel. When antimycin A was used as an inhibitor it was dissolved in ethanol and added in 0.01ml of ethanol to give a final concentration of 3μM in the experimental vessel and 0.01ml of ethanol was added to the control experiment. Results were recorded on a Rikandenki model B241 two-pen recorder. The electrode was calibrated as described by Cockburn *et al.* (1968).

Enzyme assays

Spectrophotometric assays were carried out in silica cuvettes (3.0ml volume, 1cm light-path) with a Gilford Series 2000 recording spectrophotometer.

Glycollate dehydrogenase and glutamate–glyoxylate aminotransferase (EC 2.6.1.4) were assayed as described previously (Lord & Merrett, 1970), succinate dehydrogenase (EC 1.3.99.1) as described by Lord & Merrett (1971) and malate dehydrogenase (EC 1.1.1.37) as described by Davis & Merrett (1973).

Fumarate hydratase (*EC* 4.2.1.2). This enzyme was assayed by the method of Massey (1955) which records the increase in extinction at 250nm resulting from the conversion of L-malic acid into fumaric acid. The reaction mixture contained in a final volume of 3.0ml, 100μmol of potassium phosphate buffer, pH7.4, and cell extract. The reaction was started by the addition of 50μmol of L-malate.

Hydroxypyruvate reductase (*glycerate dehydrogenase*; *EC* 1.1.1.29). This enzyme was determined by measuring the decrease in extinction consequent on the oxidation of NADH by hydroxypyruvate. The reaction mixture contained in a final volume of 3.0ml, 100μmol of Hepes buffer, pH6.5, 1μmol of NADH and cell extract. The reaction was started by the addition of 50μmol of hydroxypyruvate. No NADH was oxidized in a control cuvette containing all components except hydroxypyruvate.

Serine–glyoxylate aminotransferase (*EC* 2.6.1.45). This enzyme was assayed by the method of Brock *et al.* (1970) which involves coupling the reduction of the product hydroxypyruvate, catalysed by an excess of hydroxypyruvate reductase, to the oxidation of

* Abbreviation: Hepes buffer, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

NADH determined spectrophotometrically at 340nm. The reaction mixture contained in a final volume of 3.0ml, 100μmol of Hepes buffer, pH7.0, 1μmol of NADH, 0.1μmol of pyridoxal, 1μmol of glyoxylate, 0.05unit of hydroxypyruvate reductase and cell extract. The reaction was started by the addition of 20μmol of L-serine and the increased rate of NADH oxidation was attributed to the reduction of hydroxypyruvate produced by the serine–glyoxylate transaminase reaction.

Aspartate–α-oxoglutarate aminotransferase (*EC* 2.6.1.1). This enzyme was assayed by linking the reduction of the product, oxaloacetate, to the oxidation of NADH, catalysed by the addition of excess of malate dehydrogenase. The reaction mixture contained in a final volume of 3.0ml, 100μmol of Hepes buffer, pH7.0, 1μmol of NADH, 30μmol of α-oxoglutarate, 0.1μmol of pyridoxal phosphate, 2units of malate dehydrogenase and cell extract. The reaction was started by the addition of 20μmol of aspartate.

Isocitrate dehydrogenase (*EC* 1.1.1.41). NAD^+-dependent isocitrate dehydrogenase was assayed by measuring an increase in extinction at 340nm consequent on the reduction of NAD^+ by *threo*-D_s-isocitrate. The reaction mixture contained in a total volume of 3.0ml, 100μmol of potassium phosphate buffer, pH7.4, 30μmol of $MgCl_2$, 6μmol of GSH, 1μmol of NAD^+ and cell extract. The reaction was started by the addition of 20μmol of isocitrate.

Citrate synthase (*EC* 4.1.3.7). This enzyme was assayed as described by Weitzman (1969); the increase in extinction at 400nm was followed after the addition of 0.1μmol of oxaloacetate to a cuvette containing 9.5μmol of Tris–HCl buffer, pH8.0 0.1μmol of 5,5′-dithiobis-(2-nitrobenzoic acid), 0.15μmol of acetyl-CoA and cell extract to a total volume of 1ml.

D-*Lactate dehydrogenase* (*EC* 1.1.1.28). This enzyme was assayed by following the decrease in extinction at 600nm consequent on the reduction of 2,6-dichlorophenol-indophenol by D-lactate. The reaction mixture contained, in a total volume of 3.0ml, 100 μmol of potassium pyrophosphate buffer, pH 8.7, 0.15 μmol of 2,6-dichlorophenol-indophenol and cell extract. The reaction was started by the addition of 12.5 μmol of D-lactate.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951) by using a standard curve prepared for crystalline bovine serum albumin.

Chlorophyll determination

Chlorophyll was determined by the method of Arnon (1949).

Other materials

Nicotinamide nucleotides, pyridoxal phosphate, tetrahydrofolate, malate dehydrogenase, hydroxypyruvate reductase were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. All other materials were obtained from BDH Chemicals, Poole, Dorset, U.K., and were of the highest purity commercially available.

Results

Separation of Euglena organelles by sucrose-density-gradient centrifugation

After centrifugation of broken-cell suspension of *Euglena* on a linear sucrose gradient the distribution of specific marker enzymes for organelles was determined (Fig. 1). Hydroxypyruvate reductase

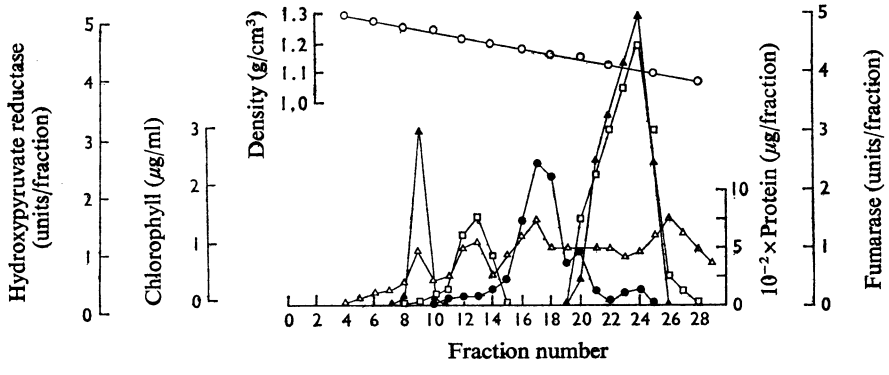

Fig. 1. *Distribution of microbody and mitochondrial marker enzymes on a continuous sucrose gradient*

Enzymes were assayed as described in the Materials and Methods section. All results are expressed as units where 1unit is 1μmol of substrate transformed/h per fraction. ▲, Hydroxypyruvate reductase; ●, chlorophyll; △, protein; □, fumarase; ○, density.

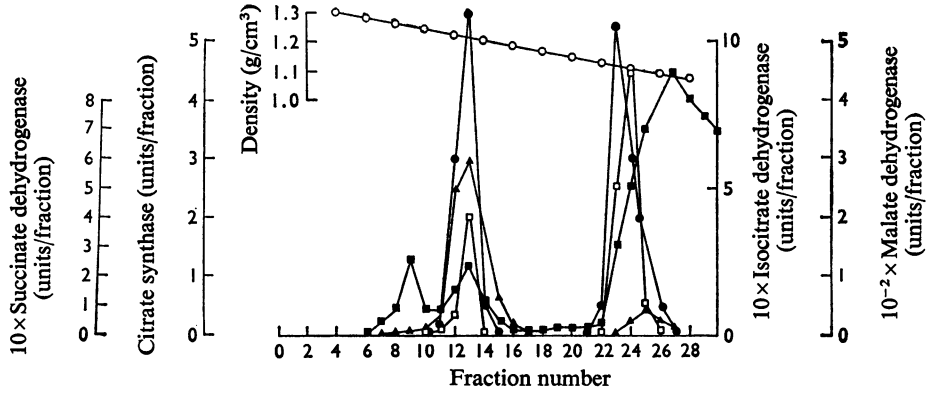

Fig. 2. *Distribution of mitochondrial enzymes from Euglena on a continuous sucrose gradient*

The enzymes were assayed as described in the Materials and Methods section. Unit is defined as μmol of substrate transformed/h per fraction. ▲, Succinate dehydrogenase; □, citrate synthase; ●, isocitrate dehydrogenase; ■, malate dehydrogenase; ○, density.

was selected as a peroxisomal marker enzyme and a major peak of activity was recorded in fraction 9 (Fig. 1) corresponding to an equilibrium density of 1.25 g/cm³. Fumarase was assayed as a marker for mitochondria and a peak of activity was present in fraction 13 at an equilibrium density of 1.22 g/cm³. Chlorophyll was measured, to record the distribution of chloroplasts and chloroplast fragments; a peak of chlorophyll was present in fraction 17, at an equilibrium density of 1.17 g/cm³. Peaks of protein were present in fractions 9, 13 and 17, corresponding to the peaks for the selected markers for peroxisomes, mitochondria and chloroplasts. After centrifugation the average recoveries of total activity added to the gradient found in various fractions were 60% for hydroxypyruvate reductase, 61% for fumarase and 93% for protein.

Distribution of tricarboxylic acid-cycle enzymes on a sucrose density gradient

To characterize further fraction 13, which gave a peak of fumarase activity, additional enzymes of the tricarboxylic acid cycle were assayed over a sucrose density gradient. Citrate synthase, succinate dehydrogenase, isocitrate dehydrogenase and malate dehydrogenase were assayed and all gave peaks of activity in fraction 13 (Fig. 2). Citrate synthase and isocitrate dehydrogenase also gave sharp peaks of activity in fractions 23 and 24 respectively which could have resulted from the damage of mitochondria during homogenization with the resultant release of enzyme. In addition to being present in fraction 13 and in the upper fractions of the gradient, malate dehydrogenase activity was present in fraction 9, locating the peroxisome-like particles, which contain a characteristic isoenzyme of malate dehydrogenase in *Euglena* (Davis & Merrett, 1973).

The assay of mitochondrial enzymes on the gradient revealed that contamination of the peroxisomal fraction by mitochondrial enzymes was minimal, succinate dehydrogenase and fumarase were barely detectable and the other specifically mitochondrial enzymes were not detected in the peroxisome fraction. The percentages of each enzyme recovered after sucrose-density-gradient centrifugation were malate dehydrogenase 91%, succinate dehydrogenase 85%, NAD^+-isocitrate dehydrogenase 67% and citrate synthase 58% respectively of the total amount added.

Subcellular location of aminotransferases in Euglena

The distribution of aminotransferases on a linear sucrose gradient was determined (Fig. 3), and, excluding enzyme activity present in the upper fractions representing non-particulate enzyme, the only peak of serine–glyoxylate aminotransferase activity present in a particulate fraction was in fraction 9, showing this enzyme to be located exclusively in the peroxisomes. Glutamate-glyoxylate aminotransferase gave two peaks of activity on the lower part of the gradient in fractions 9 and 13, the peroxisomal and mitochondrial fractions respectively. Aspartate–α-oxoglutarate aminotransferase activity was recorded in the peroxisomal, mitochondrial and chloroplast fractions (Fig. 3). Although the activity of the chloroplast fraction was far less than in the mitochondrial and peroxisomal fractions it was always detected. Overall recoveries of 91 and 65% for aspartate–α-oxoglutarate aminotransferase and serine–glyoxylate aminotransferase respectively

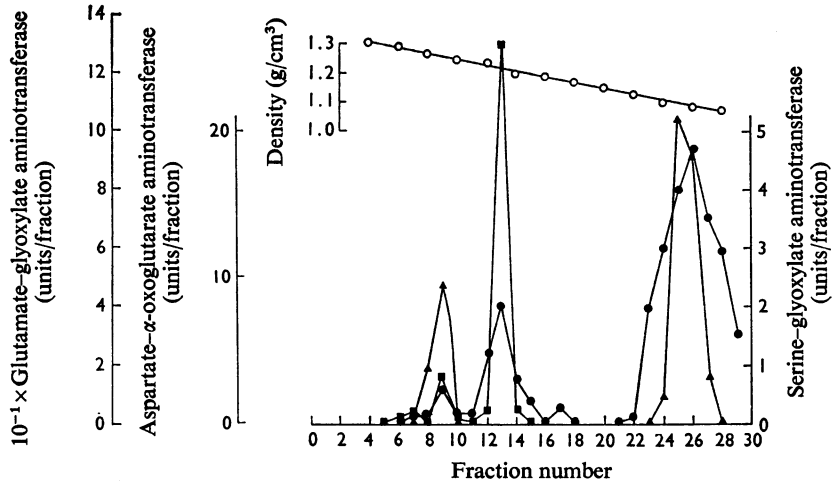

Fig. 3. *Distribution of some aminotransferases from Euglena on a continuous sucrose gradient*

The enzymes were assayed as described in the Materials and Methods section. Unit is defined as μmol of substrate transformed/h per fraction. ■, Glutamate–glyoxylate aminotransferase; ●, aspartate–α-oxoglutarate aminotransferase; ▲, serine–glyoxylate aminotransferase; ○, density.

were obtained after sucrose-gradient centrifugation. The presence in the peroxisomal fraction of some succinate dehydrogenase and fumarase activities, both mitochondrial enzymes, suggests the possibility that some or all of the aminotransferase activity in the peroxisomal fraction may have resulted from the presence of mitochondrial aminotransferases. Percentage contamination was determined by using the amount of specific marker enzymes present in each of the organelle fractions. Thus the ratio

$$\frac{\text{(Succinate dehydrogenase in 'peroxisomes')}}{\text{(succinate dehydrogenase in mitochondria)}} \times \frac{\text{(aminotransferase in mitochondria)}}{\text{(aminotransferase in 'peroxisomes')}} \times 100$$

was used to determine the percentage of the total aminotransferase activity present in the peroxisome fraction resulting from mitochondrial enzymes. By using this method, with its obvious limitations, it was found that not more than 11% of aspartate–α-oxoglutarate aminotransferase and 40% of glutamate–glyoxylate aminotransferase activity in the peroxisomal fractions could have resulted from the presence of mitochondrial enzymes. Neither succinate dehydrogenase nor any other mitochondrial enzyme was detected in the chloroplast fraction. It was therefore concluded that the chloroplast fraction was free from mitochondrial contaminants and that the relatively low aspartate–α-oxoglutarate aminotransferase activity was due to a specific chloroplast isoenzyme. This conclusion was supported by the observation that

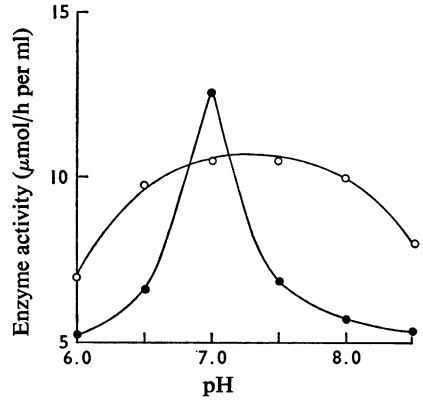

Fig. 4. *Effect of pH on aspartate–α-oxoglutarate aminotransferase activity in the mitochondrial and chloroplast fractions of Euglena after separation on a continuous sucrose gradient*

●, 10× Enzyme activity in chloroplast fraction; ○, enzyme activity in mitochondrial fraction.

the chloroplast enzyme showed a sharp peak of optimum activity at pH 7.0 whereas the mitochondrial enzyme gave a broad peak of maximum activity over the range pH 6.5 to 8.0 (Fig. 4); moreover, activity was not present in fraction 17 with gradients prepared from extracts of dark-grown *Euglena* cells lacking chloroplasts.

Table 1. *L-Serine–keto acid aminotransferase reactions in the peroxisomal fraction of Euglena*

For details see the text.

Keto donor	Rate of reaction (μmol of substrate/h per ml of peroxisomal fraction)
0.3 mM-Glyoxylate	3.7
1 mM-Pyruvate	1.7
1 mM-α-Oxoglutarate	2.0
0.3 mM-Glyoxylate +1 mM-pyruvate	2.8

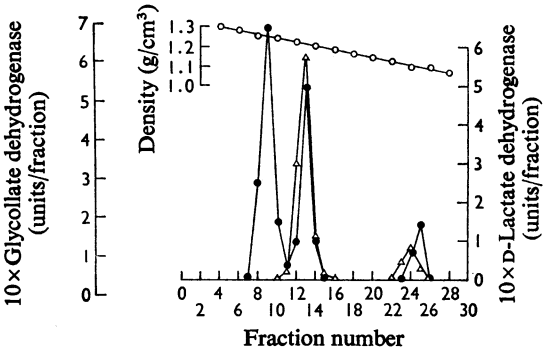

Fig. 5. *Distribution of D-lactate dehydrogenase and glycollate dehydrogenase from Euglena on a continuous sucrose gradient*

The enzymes were assayed as described in the Materials and Methods section. Unit is defined as μmol of substrate transformed/h per fraction. ●, Glycollate dehydrogenase; △, D-lactate dehydrogenase; ○, density.

The peroxisomal fraction also catalysed a serine–pyruvate aminotransferase reaction at about half the rate of the serine–glyoxylate aminotransferase reaction and a reaction also occurred with α-oxoglutarate as the keto donor (Table 1). Rates in the presence of both glyoxylate and pyruvate were not additive, suggesting that the reactions were catalysed by one enzyme.

Distribution of glycollate dehydrogenase on a sucrose-density gradient

The first enzyme of the glycollate pathway in algae, glycollate dehydrogenase, catalyses the oxidation of glycollate to glyoxylate. Peaks of activity of this enzyme were found in fraction 9, the peroxisomal fraction and fraction 13, the mitochondrial fraction (Fig. 5). The presence of a glycollate-oxidizing enzyme in the mitochondrial fraction suggested the possibility that some glycollate might also be oxidized by a non-specific L-α-hydroxyacid dehydrogenase. However, no reaction was observed with L-lactate as substrate although a peak of D-lactate dehydrogenase activity was detected in the mitochondrial fraction with D-lactate. In the presence of 1 mM-cyanide, glycollate oxidation by the mitochondrial fraction was completely inhibited, but only a 10% decrease in the rate of D-lactate oxidation was observed. Thus a cyanide-resistant D-lactate dehydrogenase and a cyanide-sensitive glycollate dehydrogenase were both present in the mitochondrial fraction; confirmatory evidence for this was that the rates of glycollate and D-lactate oxidation were additive in this fraction (Table 2). Recoveries for D-lactate dehydrogenase were 80% and glycollate dehydrogenase 91%.

Glycollate-linked oxygen uptake

The presence of glycollate dehydrogenase in the mitochondrial fraction raised the possibility that glycollate electrons were transferred to oxygen via the components of the mitochondrial electron-transport chain. Mitochondria prepared in bulk by rapid differential centrifugation were used to demonstrate a glycollate-dependent oxygen uptake that was abolished by cyanide or antimycin A (Table 3). Initially, difficulty was encountered in demonstrating oxygen uptake by mitochondria isolated by sucrose-density-gradient centrifugation. However, when the period of centrifugation was decreased to 30 min a mitochondrial fraction was obtained that metabolized a variety of substrates, including glycollate, with concomitant oxygen uptake. It was not possible to demonstrate oxygen uptake linked to glycollate oxidation with the peroxisomal fraction from the gradient.

Discussion

Microbodies, organelles characterized by a single limiting membrane and a granular matrix, have been observed in a number of unicellular green algae.

Table 2. *Rates of glycollate dehydrogenase and D-lactate dehydrogenase activity in the mitochondrial fraction of Euglena*

For details see the text.

Substrate concentration	Rate of reaction (μmol of substrate/h per ml of mitochondrial fraction)
4 mM-Glycollate	0.55
4 mM-D-Lactate	0.55
4 mM-D-Lactate+4 mM-glycollate	1.10
4 mM-D-Lactate+4 mM-glycollate +1 mM-cyanide	0.44
4 mM-D-Lactate+1 mM-cyanide	0.50
4 mM-Glycollate+1 mM-cyanide	0.01

Table 3. *Rates of glycollate-linked oxygen uptake in the mitochondrial fraction from Euglena*

For details see the text.

Substrate	O_2 uptake (nmol/min per mg of protein)	
	Mitochondria from differential centrifugation	Mitochondria from gradient
Endogenous	0.10	0.10
40mM-Glycollate	1.90	19.40
100mM-D-Lactate	0.70	4.80
40mM-Glycollate+ 3mM-antimycin A	0.04	—
40mM-Glycollate+ 0.01% ethanol	1.90	—
40mM-Glycollate+ 1mM-cyanide	0.10	—

In higher plants microbodies are of at least two types characterized by different enzyme complements, but of similar morphology. The microbodies of leaves (peroxisomes) contain enzymes of the glycollate pathway (Tolbert, 1971) whereas microbodies present in fatty seeds (often termed glyoxysomes) contain the enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase (Breidenbach & Beevers, 1967; Breidenbach *et al.*, 1968). As in higher plants it is probable that the enzyme complement of microbodies in algae varies with the growth conditions. Glyoxylate-cycle enzymes were selected as microbody markers for the characterization of microbodies from *Euglena* cells grown heterotrophically on acetate (Graves *et al.*, 1972; Bégin-Heick, 1973) and glycollate dehydrogenase as a marker for microbodies from phototrophic cells (Lord & Merrett, 1971; Davis & Merrett, 1973). Although Graves *et al.* (1972) obtained a separation of glyoxylate-cycle and mitochondrial marker enzymes by centrifuging *Euglena* extracts on a linear sucrose gradient, Bégin-Heick (1973) was unable to obtain a separation on a stepped sucrose gradient. However, results from the types of gradient are not strictly comparable, as shown by the different distribution of peroxisomal marker enzymes from phototrophic *Euglena* cells on the two types of gradient. On a stepped sucrose gradient peroxisomal malate dehydrogenase and glycollate dehydrogenase remain mainly at the 1.00M–1.25M-sucrose interface, above the heavy mitochondria at the 1.50M–1.75M-sucrose interface (Davis & Merrett, 1973), whereas on a continuous sucrose gradient peroxisomal markers equilibrate at a buoyant density of 1.25g/cm^3 which was below the light mitochondria at a buoyant density of 1.22g/cm^3 (Fig. 1). One possible explanation for this may be a differential sucrose uptake as the organelles pass down the gradient, leading to a difference in the isopycnic density of peroxisome-like particles on the two types of gradient. If therefore in a discontinuous gradient the particles are buoyant on a layer of 1.25M-sucrose they may well equilibrate within the 1.00M-sucrose environment and hence remain stuck at the 1.25M–1.00M-sucrose interface. In a linear gradient the particles can continuously equilibrate with an increasing external sucrose concentration and eventually fully equilibrate at their 'true' buoyant density.

The separation of organelles from phototrophic *Euglena* cells on a linear sucrose gradient shows that the microbody marker enzymes band together at an equilibrium density of 1.25g/cm^3, the mitochondria at an equilibrium density of 1.22g/cm^3, and the chloroplasts at an equilibrium density of 1.17g/cm^3 (Fig. 1). The possible cross-contamination between fractions was estimated by assaying for specific marker enzymes in each fraction. It was found that only membrane-bound enzymes that were probably released as organelle fragments were present as contaminants, presumably because these fragments were trapped in other organelle fractions. With enzymes present in both the peroxisomal and mitochondrial fractions, such as glutamate–glyoxylate aminotransferase and aspartate–α-oxoglutarate aminotransferase, the use of succinate dehydrogenase to determine mitochondrial contamination of the peroxisomal fraction will only give the theoretical maximum degree of contamination because if the enzymes were easily solubilized (unlike succinate dehydrogenase) the degree of cross-contamination would be less. Alternatively, the possibility was considered that some enzyme activity in the mitochondrial fraction was the result of trapping peroxisomal enzymes. In the absence of catalase from phototrophic *Euglena* cells (Lord & Merrett, 1971) only two enzymes, hydroxypyruvate reductase and serine–glyoxylate aminotransferase were specifically present in the peroxisomal fraction and neither enzyme was detected in the mitochondrial fraction. Glycollate dehydrogenase was tightly bound in the mitochondrial fraction and so did not result from the adsorption of enzyme from the peroxisomal fraction.

The fractionation of plant microbodies (Brown *et al.*, 1974) gives three distinct groups of proteins; readily soluble (matrix enzymes), soluble in the presence of KCl (membrane-bound enzymes) and relatively insoluble (membrane 'ghost' proteins). The release of enzymes from microbodies during isolation depends on the association of the enzyme within the intact organelle, enzymes readily solubilized such as hydroxypyruvate reductase (Fig. 1) being presumably matrix enzymes. The fragility of *Euglena* microbodies, resulting in loss of matrix enzymes, probably explains why in preliminary experiments attempts to prepare enriched microbody

and mitochondrial fractions by centrifuging down these organelles resulted in almost complete solubilization of some microbody marker enzymes. As this procedure was used by Graves *et al.* (1972) it may explain why they obtained such a low yield of particulate enzymes and this must throw some doubt on their conclusion that *Euglena* microbodies have an equilibrium density of 1.20g/cm^3 and mitochondria an equilibrium density of 1.17g/cm^3.

The term 'leaf peroxisome' was adopted by analogy with liver peroxisomes which are microbodies containing catalase and having a gluconeogenic function (de Duve, 1969). Catalase is required in leaf peroxisomes to destroy H_2O_2 generated by glycollate oxidase during the oxidation of glycollate to glyoxylate, this being the first reaction of the glycollate pathway which is gluconeogenic and located mainly in leaf peroxisomes (Tolbert, 1971). In *Euglena* the enzyme that oxidizes glycollate to glyoxylate does not utilize molecular oxygen as an immediate electron acceptor (Codd *et al.*, 1969; Nelson & Tolbert, 1970) so H_2O_2 is not produced and catalase is not required and is not present in *Euglena* (Lord & Merrett, 1971). Except for this the enzyme complement of the peroxisome-like fraction from *Euglena* closely parallels that of higher plant peroxisomes. Both glycollate dehydrogenase and glutamate–glyoxylate aminotransferase were present in the peroxisomal fraction, but not exclusively in this fraction as is so in higher plants (Rehfeld & Tolbert, 1972). Serine–glyoxylate aminotransferase, located exclusively in the peroxisomes, and aspartate–α-oxoglutarate aminotransferase present in 'peroxisomes', mitochondria and chloroplasts show the same subcellular distribution in *Euglena* as in spinach leaves (Rehfeld & Tolbert, 1972). Thus the enzyme complement of peroxisome-like particles from *Euglena* does not differ markedly from higher-plant peroxisomes and it is probable that the same reaction sequence operates as has been postulated to occur in higher-plant peroxisomes (Tolbert, 1971).

Glycollate is the substrate for photorespiration, an energetically wasteful process in higher plants. Although little is known about photorespiration in algae (Merrett & Lord, 1973) the presence of glycollate dehydrogenase and glutamate–glyoxylate aminotransferase in the mitochondria of *Euglena* may mean that photorespiration in algae in terms of energy yield is not a wasteful process. In higher plants only the conversion of glycine into serine occurs in the mitochondria and is linked to ATP formation (Bird *et al.*, 1972). In algae, there is the possibility of generating ATP, via the reactions of the mitochondrial electron-transport chain, during the oxidation of glycollate in addition to any ATP that might result from the subsequent metabolism of glycine as in higher plants.

We thank the Science Research Council for financial support under Grant no. B/RG/24209.

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