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Localization of Enzymes of Glycolate Metabolism in the Alga *Chlorogonium elongatum*[1]

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ABSTRACT

Organelles in homogenates from autotrophic cells of *Chlorogonium elongatum* were separated on linear sucrose gradients. The distribution of enzymes typical of leaf peroxisomes was determined.

Whereas more than 60% of the catalase activity was particulate and recovered in microbodies at a mean density of 1.225 g/cm³ within the gradient, in most experiments only 5 to 10% (as a maximum 30%) of the NAD-dependent hydroxypyruvate reductase was particulate, and this was recovered principally at density 1.19 g/cm³. This distribution coincides with that of cytochrome oxidase, malate dehydrogenase, and isocitrate dehydrogenase, the mitochondrial markers. Glyoxylate-glutamate aminotransferase and glycolate dehydrogenase showed a similar distribution pattern to that of NAD-dependent hydroxypyruvate reductase. Thus in *Chlorogonium* the enzymes of the glycolate pathway are not associated with the microbodies that are recovered at density 1.225 g/cm³.

The single large chloroplasts of the *Chlorogonium* cells are broken during grinding, and this probably accounts for the finding that NADP-glyoxylate reductase was recovered only in the soluble fractions of the gradient.

The alga *Chlorogonium elongatum* is a flagellate which is able to grow rapidly on acetate in the dark or autotrophically in the light. In accordance with the expected role of the glyoxylate cycle during growth on acetate, the activity of isocitrate lyase in heterotrophic cells is some 10-fold higher than when the cells are cultured autotrophically (11). Although microbodies were present in such cells they did not contain isocitrate lyase or malate synthetase and thus they are not glyoxysomes (13, 14).

In green cells of leaves another class of microbodies, the leaf peroxisomes, contain most of the enzymes of the glycolate pathway (15). This pathway is also active in autotrophic *Chlorogonium* cells; the enzymes have been detected (13), and the cells metabolize glycolate with concomitant release of CO_2 (12). The experiments in this paper were carried out to determine the intracellular location of enzymes concerned with glycolate metabolism in such cells.

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MATERIALS AND METHODS

Algal Material and Growing Conditions. In all experiments, strain 2E of the flagellate *Chlorogonium elongatum,* obtained from the Algensammlung des Pflanzenphysiologischen Instituts der Universität Göttingen, was used. The cells were grown autotrophically as described earlier (14). No CO_2 was added to the air. As a light source, four fluorescent lamps were used.

Preparation of Cell Homogenates. The algae in 1 liter of suspension were collected by low speed centrifugation and, after washing with distilled water the cells were suspended in 4 ml of sucrose containing grinding medium described by Gerhardt and Beevers (4). To break the cells, they were ground gently with sand in a mortar for 2 min at 0 C. Whole cells and large particles were removed from the homogenate by centrifugation at 250*g* for 8 min. Then this crude homogenate was placed on a linear gradient from 30 to 60% (w/w) sucrose. After centrifugation for 4 hr at 60,000*g*, the gradient was fractionated and enzyme activities were determined. The sucrose concentration of each fraction of the gradient was measured with a Bausch and Lomb refractometer.

Enzyme Assays. All enzyme activities except that of glyoxylate-glutamate aminotransferase were determined spectrophotometrically in a Zeiss PMQ II spectrophotometer attached to a Gilford 2000 recorder. Catalase was assayed by a method described by Lück (10). The activity of Cyt *c* oxidase was measured by an assay of Hackett (8). To each sample digitonin was added as described by Feierabend and Beevers (2).

Enzymatic reduction of hydroxypyruvate or glyoxylate was assayed by oxidation of NADH or NADPH. The reaction mixture had a volume of 1 ml and contained in a final concentration: potassium phosphate buffer, pH 6.3, 25 mM; NADH or NADPH, 0.2 mM; dithiothreitol, 1 mM. When NADH was the donor, the concentration of hydroxypyruvate (lithium salt) was 1.2 mM; when NADPH was the donor, the concentration of glyoxylate was 12 mM.

For the assay of glycolate dehydrogenase reduction of the dye, DCIP[3] was determined by the increase in absorbance at 600 nm (17). In a total volume of 1 ml, the assay mixture contained: potassium phosphate buffer, pH 8.0, 0.1 M; DCIP, 100 μM; glycolate (sodium salt), 7.5 mM.

Malate dehydrogenase activity was assayed in a mixture containing potassium phosphate buffer, pH 7.5, 0.1 M; NADH, 0.2 mM; oxaloacetate, 0.4 mM. The assay used for determination of NAD- or NADP-dependent isocitrate dehydrogenase was that described by Cooper and Beevers (1).

Glutamate-glyoxylate aminotransferase activity of gradient fractions was estimated in the following mixture: potassium phosphate buffer, pH 7.5, 0.05 M; pyridoxal phosphate, 10 μM;

[3] Abbreviation: DCIP: 2,6-dichlorophenolindophenol.

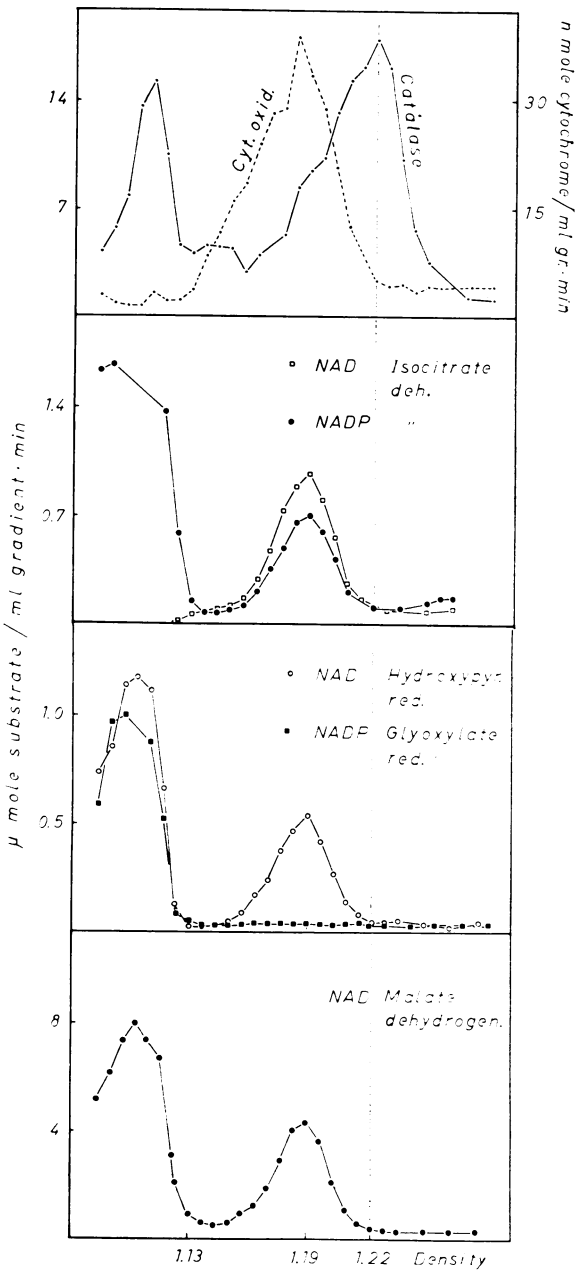

FIG. 1. Separation of organelles from the crude homogenate from autotrophic *Chlorogonium* cells in a sucrose gradient.

glutamate, 25 mM; glyoxylate, 20 mM. The reaction was stopped after 15 to 30 min by boiling in a hot water bath for 1 min. To demonstrate the glycine formed during the reaction the mixture was separated by thin layer electrophoresis. About 6 μl of the reaction mixture were applied to a silica gel thin layer plate which had a length of 20 cm and a width of 7 cm. Electrophoresis was carried out with phosphate buffer, pH 6.05, 0.05 M for 45 min. The voltage was about 100, and the intensity of current was 3 mamp. After drying, the plates were sprayed with ninhydrin reagent. The volume of 100 ml of this reagent contained: 160 mg of ninhydrin, 81 ml of ethanol, 16 ml of glacial acetic acid, 3 ml of 2,4,6-collidine, and 30 mg of $Cu(NO_3)_2 \cdot 3\ H_2O$. When the chromatograms were dried for 2 min at 100 C, glutamate appeared as violet spots, whereas glycine gave deep yellow spots which were well separated from those of glutamate.

RESULTS

As reported previously (13), clear peaks of Cyt oxidase and catalase, marking mitochondria and microbodies, occur at mean densities of 1.19 g/cm³ and 1.225 g/cm³ when the crude extract from autotrophic *Chlorogonium* cells was centrifuged in the sucrose gradient (Fig. 1). It would appear that contamination by mitochondria in the fraction at density 1.225 g/cm³ is very low.

NAD-dependent hydroxypyruvate reductase, a distinctive enzyme of leaf peroxisomes, showed only a single peak within such gradients, coinciding with the distribution of Cyt oxidase and of the additional mitochondrial markers malate dehydrogenase and the isocitrate dehydrogenases (Fig. 1). Thus the distribution of NAD-hydroxypyruvate reductase within the gradient does not match that of the main peak of catalase (and uricase [14]) the microbody markers, although, as previously reported (13), a small trailing shoulder of these activities is present in the mitochondrial region. In the experiments shown in Figure 1, about 30% of the total NAD-hydroxypyruvate reductase activity was recovered in the particulate form (*i.e.* within the gradient); in most experiments (*e.g.* Fig. 2), the recovery of particulate catalase was consistently greater than 60% of the total activity. Since both of these enzymes are readily solubilized when the cells are ground vigorously (the microbodies then should be broken), it seems that the failure to recover a significant peak of NAD-hydroxypyruvate reductase at density 1.225 g/cm³ in any of the gradients can only mean that this enzyme is not associated with the microbodies recovered at this characteristic density. The enzyme from the mitochondrial fraction or at the top of the gradient (soluble) shows a high affinity for hydroxypyruvate; maximum activity was reached at a concentration of 1.2 mM. In this regard and in the ability to reduce glyoxylate at a maximum rate only at much higher substrate concentrations (60 mM), the enzyme resembles that from higher plants (15). With pyruvate as a substrate and with NADH as coenzyme, an extremely low enzyme activity was found in the mitochondrial fraction. The activity was about one-thirtieth of that for reduction of hydroxypyruvate.

NADPH-glyoxylate reductase, an enzyme of the chloroplast stroma in higher plants (16), was present only in the soluble fraction of the gradient (Fig. 1).

Glycolate dehydrogenase activity was recovered in the gradient in only one peak at mean density 1.19 g/cm³, coinciding in distribution with that of hydroxypyruvate reductase and the mitochondrial markers (Fig. 3). The activity was completely

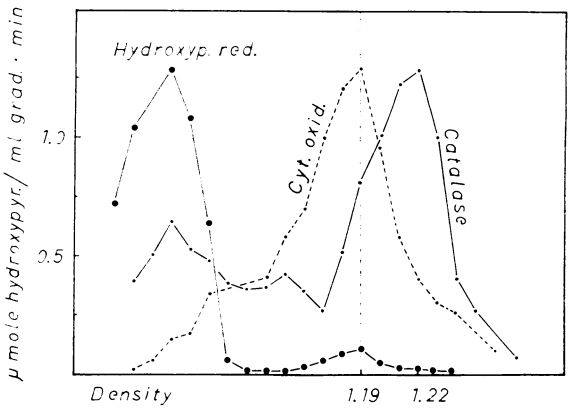

FIG. 2. Distribution of hydroxypyruvate reductase (NAD-dependent) in a gradient.

inhibited by 1 mM cyanide. It was not possible to measure activity of this enzyme in the soluble fraction of the gradient, since dithiothreitol, which is an indispensable ingredient of the extraction medium, interferes in the DCIP assay.

Glutamate-glyoxylate aminotransferase activity was present only in the soluble and mitochondrial fractions (Fig. 4). The distribution pattern of that enzyme was the same as that of malate dehydrogenase and hydroxypyruvate reductase.

DISCUSSION

When *Chlorogonium* cells are ground, there is extensive breakage of chloroplasts and mitochondria, as shown by the recovery of NADPH-glyoxylate reductase and "soluble" mitochondrial enzymes at the top of the gradient. However, consistently good yields of particulate catalase at density 1.225 g/cm³ show that intact microbodies are recovered. Nevertheless none of the enzymes concerned with the glycolate pathway, which are found together with catalase in the microbodies from leaves, was found in the microbody fraction from *Chlorogonium*. Instead, most of the hydroxypyruvate reductase and glutamate-glyoxylate aminotransferase were found in the soluble fraction and within the gradient only in the mitochondrial region. In experiments where a greater portion of the hydroxypyruvate reductase was recovered in the mitochondria band, a correspondingly greater portion of the malate dehydrogenase was also present in this fraction. It appears that the recovery of enzymes of the glycolate pathway in the mitochondrial fraction reflects the situation *in vivo*. The glycolate dehydrogenase is also present on the mitochondrial fraction, and it appears likely that its oxidation is linked to O_2 *in vivo* through the electron transport system in the mitochondria.

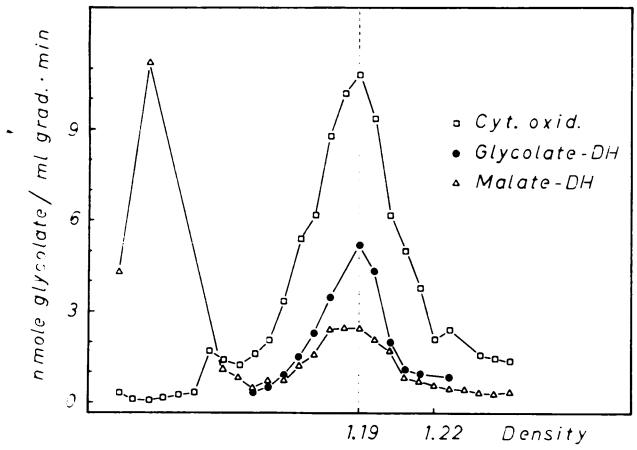

FIG. 3. Distribution pattern of glycolate dehydrogenase after separation of organelles from a crude homogenate of autotrophic *Chlorogonium* cells.

As reported by Graves *et al.* (6) enzymes from *Euglena* typical for microbodies banded at density 1.22 g/cm³. But microbodies at this density appeared to be intimately associated with mitochondria, and it seems that they move to another density when there is a better separation of these organelles (7).

The results presented here are much more similar to those for *Polytomella* (3), another acetate flagellate which is closely related to *Chlorogonium*. With that alga peaks of catalase and uricase (but not of isocitrate lyase) were also found at density 1.225 g/cm³ (3), and as shown by electron micrographs there were microbodies in the catalase containing fractions (5).

By our own electron microscopic study, we could also demonstrate microbodies in the fraction 1.225 g/cm³. These organelles did not contain crystals as reported by Gerhardt and Berger (5). Therefore it appears that catalase activity at density 1.225 g/cm³ is due to intact microbodies rather than to membrane or debris bound protein.

We interpret these and earlier results (13) to mean that the microbodies in *Chlorogonium* are of the nonspecialized type (9, 14) and house neither the enzymes of the glycolate pathway in autotrophic cells nor the enzymes of the glyoxylate cycle in cells grown on acetate (14). Instead these enzymes are found only in the soluble form (*e.g.* isocitrate lyase) or in the mitochondrial region (*e.g.* hydroxypyruvate reductase).

It is still conceivable that in *Chlorogonium* these enzymes are associated with particles of the same density as mitochondria and further work has to be done to obtain more information, but from our present observations the first interpretation is preferred.

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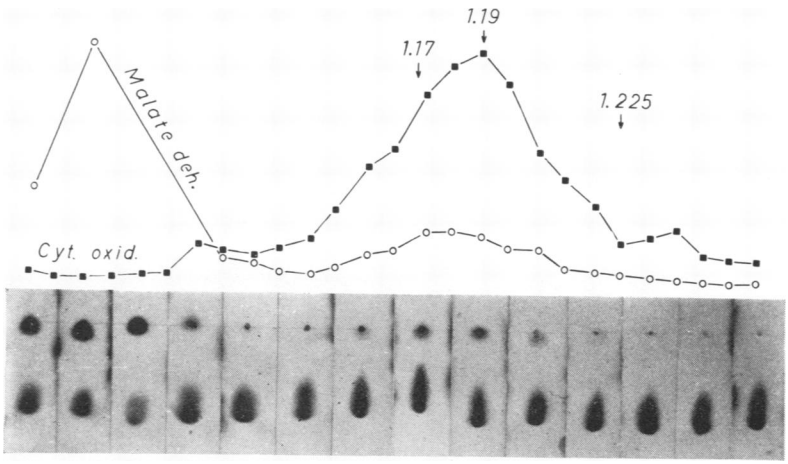

FIG. 4. Distribution of glyoxylate-glutamate aminotransferase in a gradient. To demonstrate the formation of glycine the reaction mixture was separated chromatographically after the assay. The spots in the lower line represent glutamate which was added as amino donor. Glycine did not migrate during separation, therefore spots due to this amino acid are located on the start line (upper line). Very small spots on the start line, especially around density 1.17 g/cm³, are due to chloroplast material in the fractions, which was inevitably applied to the chromatograms with the amino acids. The peak due to malate dehydrogenase on the left represents the top fractions and that due to Cyt oxidase (density 1.19 g/cm³) represents the mitochondria band of the gradient. No glycine spots were seen at density 1.225 g/cm³ (microbody band).

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