The Localization of Enzymes of Intermediary Metabolism in *Astasia* and *Euglena*

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Results are presented on the intracellular localization of some of the enzymes of gluconeogenesis, of the tricarboxylic acid cycle and of related enzymes in *Astasia* and *Euglena* grown with various substrates. The results indicate the particulate nature of at least part of the malate synthase of *Astasia* and of part of the malate synthase and isocitrate lyase in *Euglena*. However, the presence of glyoxysomes (microbodies) in *Astasia* and *Euglena* is still open to question, since it has not, so far, been possible to separate the enzymes of the glyoxylate cycle from succinate dehydrogenase in the particulate fraction.

Since the pioneering work of Kornberg and his associates (Kornberg, 1961), it has been accepted that organisms that can grow on compounds such as acetate and ethanol do so by the anaplerotic reactions of the glyoxylate cycle. Further work by Hogg & Kornberg (1963) showed that in cultures of *Tetrahymena* that are actively transforming fatty acids into carbohydrates, the enzymes specific to the glyoxylate by-pass are localized in a particulate fraction different from the mitochondria.

In the past few years, a great deal of interest has been generated with regard to the intracellular localization of the enzymes of the glyoxylate by-pass in many systems including castor bean endosperm (Breidenbach & Beevers, 1967; Breidenbach *et al.*, 1968; Cooper & Beevers, 1969; Beevers, 1969), *Tetrahymena* (Mueller *et al.*, 1968), yeast (Szabo & Avers, 1969; Duntze *et al.*, 1969; Perlman & Mahler, 1970) and *Neurospora* (Korb *et al.*, 1969; Flavell & Woodward, 1971).

Heinrich & Cook (1967) reported that some of the malate synthase activity in *Euglena gracilis* strain Z was particulate, and Graves *et al.* (1971a) reported the presence of microbodies as a result of electron microscopic studies of *Euglena gracilis*. Biochemical studies from the same group (Graves *et al.*, 1971b) indicated the particulate nature of the glyoxylate-cycle enzymes in *Euglena*. However, because no mitochondrial marker was measured the nature of the particulate fraction is still open to question.

I now present results on the intracellular localization of some gluconeogenic, tricarboxylic acid-cycle and other enzymes in *Astasia* and *Euglena*.

Materials and Methods

The cells used were *Astasia longa* (Jahn strain) and *Euglena gracilis* SMLI, a streptomycin-bleached strain. The cells were grown axenically in Cramer-Myers medium as previously described (Bégin-Heick & Blum, 1967; Blum & Bégin-Heick, 1967). The C source was either 0.02M-acetate or 0.05M-ethanol for *Astasia* and 0.065M-acetate or 0.02M-glucose for *Euglena*. The substrate used in each type of experiment is indicated with the appropriate figure or table.

The cells were counted with a Model F Coulter counter (Coulter Co., Hialeah, Fla., U.S.A.) after appropriate dilution of the sample with a medium consisting of Bouin's fixative-diluted 1:50 with 0.5% NaCl.

Preparation of cell-free extracts

The cells were harvested in mid-exponential phase at 5000 rev./min with the continuous-flow system of the Sorvall centrifuge (Ivan Sorvall, Norwalk, Conn., U.S.A.). The cell pellet was washed once with a medium containing 0.25M-sucrose, 25mM-Tris and 0.5mM-EDTA adjusted to pH 7.0 with HCl. The cells were then suspended at a concentration of 1:1 (w/v) in 0.3M-sucrose-25mM-2-(N-2-hydroxyethyl)piperazine-N'-yl)ethanesulphonic acid (Hepes)-0.5mM-EDTA adjusted to pH 7.0 with NaOH. The cell suspension was then added to silicone-coated glass beads (2.5 g/g wet wt. of cells) and the cells were disrupted for 15 to 20 s at top speed in the Braun MSK cell homogenizer at 4°C. To ensure that this treatment was not too harsh, in some of the experiments the cells were disrupted by grinding by hand (Bégin-Heick & Blum, 1967). With the latter method the results were qualitatively

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similar to the results obtained with the homogenizer but they were less reproducible.

The homogenate was decanted from the glass beads and the beads were washed several times with portions of sucrose–Hepes–EDTA medium. The final homogenate was then adjusted to 20% (w/v).

Fractionation of the homogenate

The crude homogenate was centrifuged at 1200g for 10min to remove cellular debris, glass beads and the bulk of the paramylon. The resulting fraction (S_1) was then centrifuged at 10500g for 15min to yield a supernatant fraction (S_2) essentially free of mitochondria, and a particulate fraction (P_1) containing the mitochondria. Fraction S_2 was further centrifuged at 100000g for 30min to give supernatant S_3 .

The particulate fraction (P_1) was washed once and resuspended in the sucrose–Hepes–EDTA medium at a protein concentration of 8–10mg/ml.

Fractionation of the particulate fraction

The crude mitochondrial pellet was fractionated by centrifugation through a stepped sucrose-density gradient of the following (w/v) composition: 60% sucrose, 3ml; 57% sucrose, 5ml; 50% sucrose, 9.5ml; 44% sucrose, 8.5ml and 32% sucrose, 3ml. The crude particulate suspension (2ml) was layered on top of the gradient. The tubes were centrifuged for 4.5h at 25000rev./min in a SW25 rotor in a Beckman model L centrifuge. The gradient was fractionated by puncturing the tube bottom and collecting 20-drop fractions.

Enzyme assays

The enzyme assays were done on the fractions (S_1 , S_2 , S_3 and P_1) and also on the fractions from the sucrose gradients. All spectrophotometric assays were performed on a Gilford model 2000 spectrophotometer (Gilford Instruments, Oberlin, Ohio, U.S.A.) at 26°C. The assays were done at two protein concentrations chosen so that activity was proportional to the protein concentration.

Citrate synthase (EC 4.1.3.7). The increase in E_{412} was followed after the addition of 7.5mm-oxaloacetate to a cuvette containing 90 μ mol of Tris adjusted to pH8.0 with HCl, 1mm-5,5'-dithiobis-(2-nitrobenzoic acid) and 0.15mm-acetyl-CoA and 10–100 μ g of protein in a total volume of 1ml.

Malate synthase (EC 4.1.3.2). Malate synthase was assayed as for citrate synthase except that 12mm-MgCl₂ was included in the reaction mixture and 10mm-glyoxylate was added to start the reaction instead of oxaloacetate. For both these enzymes, the specific enzyme activity was calculated by assum-

ing a molar extinction coefficient of 1.3×10^7 litre·mol⁻¹·cm⁻¹ (Ellman, 1959).

Isocitrate lyase (EC 4.1.3.1). The activity was determined by the method of Dixon & Kornberg (1959). The reaction was initiated by adding 3.3mm-D₆-isocitrate to 1ml of a mixture of 83mm-sodium phosphate buffer adjusted to pH6.8 with NaOH, 8mm-MgCl₂ and 15mm-phenylhydrazine. The change in E_{324} was followed and the molar extinction coefficient was determined to be 2.2×10^7 litre·mol⁻¹·cm⁻¹.

Malate dehydrogenase (EC 1.1.1.37). Malate dehydrogenase was assayed in the direction of malate to oxaloacetate. The reaction mixture contained 1.0ml of 1M-glycine–0.4M-hydrazine buffer, pH9.5, 3mm-NAD⁺ and 7mm-malate in a total volume of 3ml. The reaction was initiated by the addition of 10–30 μ g of protein. The change in E_{340} was followed.

Fumarate hydratase (fumarase) (EC 4.2.1.2). Fumarase was determined by measuring the conversion of malate into fumarate at 240nm. The reaction volume was 3ml and the reaction mixture contained 75mm-sodium phosphate buffer adjusted to pH7.4 with NaOH, 7.0mm-malate and 10–40 μ g of protein. The extinction coefficient for fumarate was assumed to be 2.6×10^3 litre·mol⁻¹·cm⁻¹ (Cooper & Beevers, 1969).

Aconitate hydratase (aconitase) (EC 4.2.1.3). The reaction was initiated by the addition of 1mm-citrate to 3ml of a reaction mixture containing 33mm-Tris adjusted to pH7.4, with HCl, 1.3mm-MgCl₂, 0.3mm-NADP⁺ and 50 μ g of NADP⁺-specific isocitrate dehydrogenase (0.2–0.5 μ molar units) and 100–300 μ g of protein. The changes in E_{340} were measured.

Pyruvate carboxylase (ADP) (EC 6.4.1.1). This was assayed in 33mm-Tris buffer adjusted to pH8.0 with HCl, essentially as described by Young *et al.* (1969). Acetyl-CoA was omitted from the reaction mixture because its addition did not increase the enzyme activity.

2-Oxo acid carboxy-lyase (EC 4.1.1.1). This was assayed by coupling the reaction with alcohol dehydrogenase. The reaction mixture contained 23mm-sodium phosphate buffer, pH6.8, 8mm-sodium pyruvate, 0.02mm-NADH and 8mm-MgCl₂ and 10 units of yeast alcohol dehydrogenase. The reaction was initiated by the addition of pyruvate.

Other enzyme assays. Aspartate aminotransferase (glutamate–oxaloacetate transaminase) (EC 2.6.1.1) and phosphoenolpyruvate carboxylase (EC 4.1.1.32) were measured as described by Cooper & Beevers (1969). Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) were assayed by the method of Cartier *et al.* (1967). NAD⁺-dependent alcohol dehydrogenase (EC 1.1.1.1) was assayed by

the method of Bonnichsen (1965). NAD⁺-dependent isocitrate dehydrogenase (EC 1.1.1.41) was assayed as described by Plaut (1969) and NADP⁺-dependent isocitrate dehydrogenase (EC 1.1.1.42) as described by Cleland *et al.* (1969). Succinate dehydrogenase (EC 1.3.99.1), 'malic' enzyme (NADP⁺-dependent malate dehydrogenase, decarboxylating) (EC 1.1.1.39) and fructose 1,6-diphosphatase (EC 3.1.3.11) were assayed as described previously (Bégin-Heick, 1970).

Dithiothreitol or glutathione did not increase the enzymic activities that were studied and β -mercaptoethanol inhibited many of these enzyme activities. These compounds were therefore left out of the reaction mixtures. Cofactors, substrates and enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. They were of the purest grade available.

The enzymic activities (except for succinate dehydrogenase) were measured before and after addition of 0.066% Triton X-100 to release any latent activity. Triton X-100 inhibited 99% of the NADH oxidase and succinate dehydrogenase activity in the system, without any deleterious effects on the other enzyme activities. It was therefore always used in reactions where NAD⁺ was a cofactor.

Protein determinations

These were done by the method of Lowry *et al.* (1951).

Results

The enzymic activities that were studied have been divided into three classes: (a) enzymes present in both the particulate fraction and in the cytosol; (b) enzymes found only in the particulate fraction; (c) enzymes found in the cytosol. All enzymes found in two locations have the following characteristics in common. (i) The removal of the particulate fraction containing the mitochondria leads to a decrease of the total activity and of the specific activity of the resulting supernatant (S₂) relative to the crude homogenate (S₁). (ii) The addition of Triton X-100 is required to reveal full activity in the crude homogenate (S₁) and in the particulate fraction (P₁) but not in the other fractions. (iii) The specific activity of the particulate form of the enzyme is equal to or greater than, the specific activity of the form present in the cytosol.

Distribution of enzyme activities in Astasia (Table 1)

The relative distribution of the enzymes in the four fractions is constant and comparable between experiments. The specific activities are somewhat more variable, and the means and standard deviations are given for all cases where four or more determinations

were available for the same enzyme. Since the aim was not to establish significant differences between enzyme activities obtained under various conditions, the fact that the standard errors are fairly large is less of a disadvantage.

(a) Enzymes with dual location

The enzymes of this group were distributed between the particulate (P₁) and the cytosol (S₃) fractions. Except for succinate dehydrogenase, all of the enzymes necessary for the operation of the tricarboxylic acid cycle that were examined fell in this category. The total activities of the enzymes of this group decreased by 20 to 55% because of the removal of the fraction containing the mitochondria. Further centrifugation to remove the microsomal material did not affect the distribution of these enzyme activities appreciably, except for aconitase and fumarase, where a large proportion of the total enzyme activity was removed by centrifugation at 100000g. These two enzymes behave in a similar manner in other systems (Greville, 1969). The activity lost from the total homogenate by removal of the particulate fraction was recovered in fraction P₁ in a proportional manner. Alcohol dehydrogenase and glutamate-oxaloacetate transaminase, which catalyse reactions leading to the tricarboxylic acid cycle, have a comparable distribution. Malate synthase, which catalyses a reaction of the glyoxylate bypass, and which may also be necessary for the metabolism of glycolate is also in this category.

(b) Particulate enzymes

Only one of the enzymes studied consistently fell in this category: succinate dehydrogenase, which was recovered entirely in the particulate fraction (P₁). In the present system, this enzyme was completely inhibited by 0.066% Triton X-100. When NADH oxidase was measured its distribution was always entirely particulate; its activity was also inhibited by Triton X-100. Glutamate dehydrogenase, which in many systems is chosen as the mitochondrial marker, was absent from *Astasia* mitochondria, as found for *Euglena* by Ammon & Friedrich (1967).

(c) Cytosol enzymes

In this category were: one of the enzymes of the glyoxylate by-pass, isocitrate lyase; two enzymes specific to gluconeogenesis from oxaloacetate: phosphoenolpyruvate carboxylase and fructose 1,6-diphosphatase; enzymes concerned with the metabolism of pyruvate: pyruvate carboxylase (ADP), 2-oxo acid carboxy-lyase and decarboxylating malate dehydrogenase (NADP⁺-linked), as well as two

Table 1. *Distribution of total enzyme activity in Astasia cultivated with ethanol as the C source*

The experimental procedures are described in the Materials and Methods section. Specific activities are expressed as nmol of substrate converted $\cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$. The standard error of the mean is given in all cases where four or more determinations were averaged. In all cases the values quoted are the means of at least three determinations. — denotes that no activity was detected.

Fraction ...	S ₁		S ₂		S ₃		P ₁	
	%	Sp. act. $\pm$ S.E.M.	%	Sp. act. $\pm$ S.E.M.	%	Sp. act. $\pm$ S.E.M.	%	Sp. act. $\pm$ S.E.M.
(a) Dually located enzymes								
Alcohol dehydrogenase	100	64.1 $\pm$ 6.2	49	41 $\pm$ 7.7	44	51.3 $\pm$ 10.4	29	95 $\pm$ 2
Citrate synthase	100	119 $\pm$ 12	79	110 $\pm$ 15	73	149 $\pm$ 17	20	154 $\pm$ 11
Malate synthase	100	144 $\pm$ 8.3	86	132 $\pm$ 23	85	156 $\pm$ 28	15	152
Aconitase	100	23.8 $\pm$ 0.95	76	22.3 $\pm$ 1.5	53	19.5 $\pm$ 1.5	13	15.4
Isocitrate dehydrogenase (NAD ⁺ -linked)	100	33.5 $\pm$ 2.3	80	31.3 $\pm$ 5.1	74	39.8 $\pm$ 7.0	10	41.0 $\pm$ 12
Malate dehydrogenase	100	698 $\pm$ 86	79	755 $\pm$ 125	75	775 $\pm$ 105	12	390 $\pm$ 50
Fumarase	100	131	45	65.1	29	59.5	42	205
Glutamate-oxaloacetate trans-aminase	100	140 $\pm$ 2	80	143 $\pm$ 13	78	163 $\pm$ 24	18	138
(b) Particulate enzymes								
Succinate dehydrogenase	100	6.5 $\pm$ 0.7	—	—	—	—	100	40.2 $\pm$ 3.0
(c) Cytosol enzymes								
Isocitrate lyase	100	27.4	110	37.8	121	47.3	2.5	1.90
Phosphoenolpyruvate carboxylase	100	28.3 $\pm$ 3	103	35.5 $\pm$ 6.0	99	46.5 $\pm$ 14	—	—
Fructose 1,6-diphosphatase	100	45.2	92	49.5	95	69.3	—	—
Isocitrate dehydrogenase (NADP ⁺ -linked)	100	50.7 $\pm$ 8.2	92	50.5 $\pm$ 11	95	73.1 $\pm$ 9	2.8	6.3
2-Oxo acid carboxy-lyase	100	45.5	94	54.5	103	71.2	—	—
Pyruvate carboxylase (ADP)	100	30.5	99	36.9	96	49.2	—	—
Malate dehydrogenase, decarboxylating (NADP ⁺ -linked)	100	192	94	213	99	270	6	64.7
Glucose 6-phosphate dehydrogenase	100	21.6 $\pm$ 0.3	107	26.2 $\pm$ 0.25	98	34.3 $\pm$ 3.3	2	6.8
6-Phosphogluconate dehydrogenase	100	22.3	109	31.5	104	41.6	—	—

enzymes of the pentose shunt: glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The NADP⁺-dependent isocitrate dehydrogenase was also in this group. The total amount of activity of these enzymes was changed very little by centrifugation of the homogenate (S₁) to remove the particulate fractions. Fractions S₂ and S₃ contained approximately the same number of units of enzyme activity as S₁. However, the specific activities of the enzymes contained in these fractions was greater in S₃ than in S₁. In most cases, no activity could be detected in fraction P₁. The small amount of activity that was sometimes found associated with P₁ can be attributed to a contamination of the particulate fraction. With isocitrate lyase, the removal of the particulate matter seemed to increase enzyme activity to such an extent that more activity was recovered in fraction S₃ than in S₁. The addition of Triton X-100

to the various fractions during the assay of these enzymes did not change any activities.

The results presented in Table 1 are for *Astasia* grown with ethanol as the C source. With *Astasia* grown on acetate, the enzyme distribution was similar to that in ethanol-grown *Astasia*, although the specific activities of some enzymes were different. Thus citrate synthase and malate synthase were both much less active in the acetate-grown *Astasia*. The specific activities (expressed in nmol of substrate converted/min per mg of protein) for these enzymes in fraction S₃ of acetate-grown cells were 31.4 and 36.4, respectively, whereas in fraction P₁ they were 25.5 and 46. On the other hand, the malate dehydrogenase activity of acetate-grown *Astasia* was twice as high as that in ethanol-grown *Astasia*. Deprivation of a C source for 48 h or O₂ treatment for 48 h did not change the distribution of these enzymes in *Astasia*.

Table 2. Distribution of enzyme activities in *Euglena* grown with acetate as the C source

The experimental details are described in the Materials and Methods section. Specific activities are expressed as nmol of substrate converted · min⁻¹ · mg of protein⁻¹. — denotes that no activity was detected.

Fraction ...	S ₁		S ₂		S ₃		P ₁	
	%	Sp. act.	%	Sp. act.	%	Sp. act.	%	Sp. act.
(a) Dually located								
Alcohol dehydrogenase	100	10.4	37	5.04	45	6.56	20	8.8
Citrate synthase	100	112	57	85.0	50	74.5	50	480
Isocitrate lyase	100	0.25	128	0.35	76	0.28	76	1.49
Malate synthase	100	100	50	66.3	49	79.2	40	192
Aconitase	100	107	33	38.7	16	23.3	33	190
Isocitrate dehydrogenase (NAD ⁺ -linked)	100	43.2	56	26.1	52	32.6	42.5	142
Malate dehydrogenase	100	822	52	533	45	554	15	738
Fumarase	100	58.5	33	23.8	35	30.3	17	59.5
Malate dehydrogenase, decarboxylating (NADP ⁺ -linked)	100	343	91	420	87	421	53	1010
(b) Particulate								
Succinate dehydrogenase	100	49	—	—	—	—	100	170
(c) Cytosol								
Phosphoenolpyruvate carboxylase	100	114	95	145	92	147	—	—
Fructose 1,6-diphosphatase	100	90.3	99	105	110	129	—	—
Isocitrate dehydrogenase (NADP ⁺ -linked)	100	112	101	130	100	166	8	14.1
2-Oxo acid carboxy-lyase	100	36.2	106	47.7	101	55.2	—	—
Pyruvate carboxylase (ADP)	100	8.96	128	13.9	92	20.3	—	—
Glucose 6-phosphate dehydrogenase	100	302	98	400	89	379	1.3	24.2
6-Phosphogluconate dehydrogenase	100	142	88	165	97	194	1.1	9.8

Distribution of enzymic activities in *Euglena*

The distribution of the same enzymes was studied in *Euglena* grown on acetate (Table 2), in which case the glyoxylate cycle operates, and on glucose (Table 3), where the glyoxylate cycle is repressed. With a few noteworthy exceptions, the distribution of the enzymic activities in acetate-grown *Euglena* was similar to that in *Astasia*. However, the specific activity of many particulate enzymes is much higher than in the corresponding fraction in *Astasia*.

Isocitrate lyase activity was present in the particulate fraction (P_1) of *Euglena*. The specific activity of this enzyme in the homogenate (S_1) was, however, much lower in *Euglena* ($0.327 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$) than in *Astasia*. In *Euglena* grown on acetate, all of the enzymes necessary for the operation of the glyoxylate by-pass were present in the particulate fraction as well as the cytosol.

The enzymes specific to the glyoxylate by-pass were repressed by growth of *Euglena* on glucose (Table 3). Isocitrate lyase activity was too low to be measured and the malate synthase activity of the cytosol (S_3) was decreased to one-twentieth of the value obtained with acetate-grown cells. The value for the particulate fraction was not decreased so drastically and remains at about one sixth of the value found when the cells grow on acetate.

NADP $^{+}$ -dependent malate dehydrogenase ('malic' enzyme) was also found in the particulate fraction of *Euglena*, but its presence could not be demonstrated in the particulate fraction of *Astasia*. In *Euglena*, the activity of the particulate form of this enzyme seems to be affected by the growth substrate, since the activity in cells grown in glucose medium was only about 70% of the activity of cells grown in acetate medium.

Table 3. Distribution of enzyme activities in *Euglena* grown with glucose as the C source

The experimental details are described in the Materials and Methods section. Specific activities are expressed as nmol of substrate converted $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. — denotes that no activity was detected.

Enzyme	Fraction ... S_1		S_2		S_3		P_1	
	%	Sp. act.	%	Sp. act.	%	Sp. act.	%	Sp. act.
(a) Dually located								
Citrate synthase	100	92.8	35	44.2	45	59.5	50	230
Isocitrate lyase	—	—	—	—	—	—	—	—
Malate synthase	100	7.50	22	2.52	31	3.35	60	29.6
Aconitase	100	60.9	50	41.0	43	53.3	59	278
Isocitrate dehydrogenase (NAD $^{+}$ -linked)	100	58.8	48	35.8	31	26.1	29	145
Malate dehydrogenase	100	880	68	800	70	823	16	1060
Fumarase	100	153	33	70.5	34	77.3	20	243
Malate dehydrogenase, decarboxylating (NADP $^{+}$ -linked)	100	180	99	250	81	320	47	680
(b) Particulate								
Succinate dehydrogenase	100	11.1	—	—	—	—	108	80.8
(c) Cytosol								
Phosphoenolpyruvate carboxylase	100	155	92	200	92	212	—	—
Fructose 1,6-diphosphatase	100	47.8	110	68.5	110	75.8	—	—
Isocitrate dehydrogenase (NADP $^{+}$ -linked)	100	119	90	128	86	138	8	80.1
2-Oxo acid carboxy-lyase	100	71.9	86	87.0	96	103	—	—
Pyruvate carboxylase (ADP)	100	76.4	75	81.2	130	148	—	—
Glucose 6-phosphate de- hydrogenase	100	186	109	261	127	338	—	—
6-Phosphogluconate de- hydrogenase	100	84.8	122	133	104	126	—	—

Fig. 1. Localization of protein and enzymic activities obtained after centrifugation of a particulate fraction from acetate-grown *Astasia* on a density gradient

Experimental details are given in the Materials and Methods section. Enzyme activities are expressed as nmol of substrate converted $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. The highest fraction number is that of the fraction at the top of the gradient.

Fractionation of particulate fractions from *Astasia* and *Euglena* on sucrose-density gradients

As some enzymes specific to the glyoxylate by-pass were present in the particulate fractions obtained from both *Astasia* and *Euglena*, these particulate fractions were fractionated to find the location of these enzymes (Figs. 1 and 2 respectively). Similar results were obtained with *Euglena* grown with acetate as the carbon source. In each case, part (a) of each figure shows the distribution of the particulate protein on the sucrose-density gradient. In both cases the protein was distributed between a major peak and a minor peak, the major peak being of higher

density. The four enzyme activities measured were present in both peaks.

The distribution of enzyme activity was measured also in the particulate fraction for *Euglena* grown on acetate. Again two protein peaks were recovered and the distribution of the enzyme activities corresponded to the protein peaks; however, both the heavier and the lighter peaks were of approximately the same magnitude (Fig. 3).

The distribution of NAD $^{+}$ -dependent isocitrate dehydrogenase activity and NADP $^{+}$ -linked malate dehydrogenase (decarboxylating) activity on these gradients was exactly the same as that of the other enzymes (results not shown).

Fig. 2. Localization of protein and enzymic activities obtained after centrifugation of a particulate fraction from glucose-grown *Euglena* on a density gradient

Experimental details are given in the Materials and Methods section. For other details see Fig. 1.

Discussion

The presence of specialized organelles containing the enzymes necessary for the transformation of acetyl-CoA into carbohydrate has been observed in several systems. In many of these systems these specialized organelles are responsible for the transformation of accumulated fatty acids into glucose.

In yeast, Szabo & Avers (1969) found malate synthase and isocitrate lyase in three fractions: mitochondria, peroxisomes and cytosol. The peroxisomes contained the catalase activity and they also contained a greater proportion of the isocitrate lyase and the malate synthase activity than the mitochondrial fraction. Perlman & Mahler (1970) found all of the isocitrate lyase activity in the cytosol, but the catalase was in the microbodies. Likewise, Duntze *et al.* (1969) reported the presence of malate synthase and isocitrate lyase in the cytosol only.

In *Neurospora* (Korb *et al.*, 1969) a portion of the glyoxylate cycle enzymes were detected in a particulate fraction that was distinguished from the mitochondria on the basis of its sedimentation behaviour. However, a greater proportion of the isocitrate lyase was found in the supernatant fraction isolated from cells grown on acetate than in the particulate fraction.

Heinrich & Cook (1967) reported the particulate nature of part of the malate synthase activity in *Euglena gracilis* strain Z, and Graves *et al.* (1971a) reported electron-microscopic studies in which they show the presence of microbodies in *Euglena*, grown on acetate or on ethanol. Unfortunately, Graves *et al.* (1971a) did not report on the fate of these microbodies in glucose-grown *Euglena*, from which they should be absent if they are identical with glyoxysomes. Graves *et al.* (1971b) found much of the glycolate dehydrogenase activity to be particulate.

Fig. 3. Localization of protein and enzymic activities obtained after centrifugation of a particulate fraction from acetate-grown *Euglena* on a density gradient

Experimental details are given in the Materials and Methods section. For other details see Fig. 1.

However, because they were unable to measure the succinate dehydrogenase activity of their fractions, they were unable to distinguish between microbodies and mitochondria. My results show that, in general, in both *Astasia* and *Euglena* the enzymes of the glyoxylate by-pass are in the particulate fraction and that a considerable proportion of these enzymes is also found in the soluble fraction, with the exception of isocitrate lyase. I could not demonstrate the presence of this enzyme in the particulate fraction of *Astasia* grown either on ethanol or on acetate.

As we were able to measure succinate dehydrogenase activity (Bégin-Heick & Blum, 1967; Blum

& Bégin-Heick, 1967) it should have been possible to distinguish between mitochondria and glyoxysomes (microbodies) if they had been separated on a gradient. However, if a continuous gradient gave a single broad and ill-defined peak that contained all the enzyme activities found in the particulate fraction, the use of a stepped gradient similar to that used by Cooper & Bevers (1969) led to the clear separation of two protein bands that both contained succinate dehydrogenase as well as the other particulate enzymes in similar proportions.

The presence of glyoxylate-cycle enzyme activities in the cytosol and in a particulate fraction that also

contains succinate dehydrogenase activity is puzzling. (a) Glyoxysomes are notoriously fragile and it could be argued that the enzyme activity recovered in the cytosol is there as a result of leaching out from broken organelles. (b) The presence of glyoxylate-cycle enzymes in the particulate fractions containing the mitochondria might be explained by a close association between mitochondria and glyoxysomes, which even centrifugation through a sucrose gradient could not break. If this were so, however, the presumed microbodies of *Astasia* and *Euglena* would be very sturdy indeed, as dilution of the particulate fraction in hypo-osmotic medium is not sufficient to release enzyme activity, and Triton X-100 must still be added to such a diluted fraction to reveal the activity of isocitrate lyase (*Euglena*) and of malate synthase (*Astasia* and *Euglena*). The integrity of the mitochondrial fraction is demonstrated by the fact that no loss of succinate dehydrogenase activity occurs during the purification of the particulate fraction.

The two techniques used for cell disruption gave comparable results. The hand-grinding method developed by Kahn & Blum (1967) gave lower amounts of particulate fraction, presumably because of much lower percentage of cell breakage. The method of disruption in the Braun MSK homogenizer was used successfully by Sharpless & Butow (1970) to produce mitochondria that gave high P/O ratios.

I am unable at this time to confirm the speculation of Graves *et al.* (1971b) that the glyoxylate-cycle enzymes are localized in microbodies. Unlike these authors, I was able to show that the particulate fraction isolated from *Astasia* and *Euglena* contains succinate dehydrogenase and must therefore contain mitochondria; however, like these authors I found no biochemical evidence for or against the presence of glyoxysomes in *Astasia* and *Euglena*.

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