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 anaerobic 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 (Bégain-Heick & Blum, 1967; Blum & Bégain-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 5000rev./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-hydroxyethylpiperazin-N'-y)ethanesulphonic acid (Hepes)–0.5mM-EDTA adjusted to pH 7.0 with NaOH. The cell suspension was then added to silicone-coated glass beads (2.5g/g wet wt. of cells) and the cells were disrupted for 15 to 20s 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 (Bégain-Heick & Blum, 1967). With the latter method the results were qualitatively
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 10 min to remove cellular debris, glass beads and the bulk of the paramylon. The resulting fraction (S1) was then centrifuged at 10500g for 15 min to yield a supernatant fraction (S2) essentially free of mitochondria, and a particulate fraction (P1) containing the mitochondria. Fraction S2 was further centrifuged at 100000g for 30 min to give supernatant S3.

The particulate fraction (P1) was washed once and resuspended in the sucrose–Hepes–EDTA medium at a protein concentration of 8–10 mg/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, 3 ml; 57% sucrose, 5 ml; 50% sucrose, 9.5 ml; 44% sucrose, 8.5 ml and 32% sucrose, 3 ml. The crude particulate suspension (2 ml) was layered on top of the gradient. The tubes were centrifuged for 4.5 h 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 (S1, S2, S3 and P1) 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 E412 was followed after the addition of 7.5 mM oxaloacetate to a cuvette containing 90 µmol of Tris adjusted to pH 8.0 with HCl, 1 mM-5,5'-dithiobis-(2-nitrobenzoic acid) and 0.15 mM-acetyl-CoA and 10–100 µg of protein in a total volume of 1 ml.

**Malate synthase (EC 4.1.3.2).** Malate synthase was assayed as for citrate synthase except that 12 mM MgCl2 was included in the reaction mixture and 10 mM-glyoxylate was added to start the reaction instead of oxaloacetate. For both these enzymes, the specific enzyme activity was calculated by assuming a molar extinction coefficient of 1.3 x 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 3.3 mM D2-isocitrate to 1 ml of a mixture of 83 mM-sodium phosphate buffer adjusted to pH 6.8 with NaOH, 8 mM-MgCl2 and 15 mM-phenylhydrazine. The change in E344 was followed and the molar extinction coefficient was determined to be 2.2 x 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 1.0 ml of 1 m-glycine–0.4 M-hydrazone buffer, pH 9.5, 3 mM-NADH± and 7 mM-malate in a total volume of 3 ml. The reaction was initiated by the addition of 10–30 µg of protein. The change in E340 was followed.

**Fumarate hydratase (fumarase) (EC 4.2.1.2).** Fumarase was determined by measuring the conversion of malate into fumarate at 240 nm. The reaction volume was 3 ml and the reaction mixture contained 75 mM-sodium phosphate buffer adjusted to pH 7.4 with NaOH, 7.0 mM-malate and 10–40 µg of protein. The extinction coefficient for fumarate was assumed to be 2.6 x 10^3 litre·mol^{-1}·cm^{-1} (Cooper & Beever, 1969).

**Aconitate hydratase (aconitase) (EC 4.2.1.3).** The reaction was initiated by the addition of 1 mM-citrate to 3 ml of a reaction mixture containing 33 mM-Tris adjusted to pH 7.4, with HCl, 1.3 mM-MgCl2, 0.3 mM-NADH± and 50 µg of NADP±-specific isocitrate dehydrogenase (0.2–0.5 pmol units) and 100–300 µg of protein. The changes in E340 were measured.

**Pyruvate carboxylase (ADP) (EC 6.4.1.1).** This was assayed in 33 mM-Tris buffer adjusted to pH 8.0 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 carboxylase (EC 4.1.1.1).** This was assayed by coupling the reaction with alcohol dehydrogenase. The reaction mixture contained 23 mM-sodium phosphate buffer, pH 6.8, 8 mM-sodium pyruvate, 0.02 mM-NADH and 8 mM-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 & Beever (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 \(\beta\)-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 (S\(_2\)) relative to the crude homogenate (S\(_1\)). (ii) The addition of Triton X-100 is required to reveal full activity in the crude homogenate (S\(_1\)) and in the particulate fraction (P\(_1\)) 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 (P\(_1\)) and the cytosol (S\(_2\)) 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 100 000g. 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 P\(_1\) 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 (P\(_1\)). 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 bypass, 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 min⁻¹ 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.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$S_1$ %</th>
<th>% Sp. act. ± S.E.M.</th>
<th>$S_2$ %</th>
<th>% Sp. act. ± S.E.M.</th>
<th>$S_3$ %</th>
<th>% Sp. act. ± S.E.M.</th>
<th>$P_1$ %</th>
<th>% Sp. act. ± S.E.M.</th>
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<td>(a) Dually located enzymes</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>100</td>
<td>64.1 ± 6.2</td>
<td>49</td>
<td>41 ± 7.7</td>
<td>44</td>
<td>51.3 ± 10.4</td>
<td>29</td>
<td>95 ± 2</td>
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<td>119 ± 12</td>
<td>79</td>
<td>110 ± 15</td>
<td>73</td>
<td>149 ± 17</td>
<td>20</td>
<td>154 ± 11</td>
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<tr>
<td>Malate synthase</td>
<td>100</td>
<td>144 ± 8.3</td>
<td>86</td>
<td>132 ± 23</td>
<td>85</td>
<td>156 ± 28</td>
<td>15</td>
<td>152</td>
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<td>Aconitase</td>
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<td>23.8 ± 0.95</td>
<td>76</td>
<td>22.3 ± 1.5</td>
<td>53</td>
<td>19.5 ± 1.5</td>
<td>13</td>
<td>15.4</td>
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<td>Isocitrate dehydrogenase (NAD⁺-linked)</td>
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<td>33.5 ± 2.3</td>
<td>80</td>
<td>31.3 ± 5.1</td>
<td>74</td>
<td>39.8 ± 7.0</td>
<td>10</td>
<td>41.0 ± 12</td>
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<td>79</td>
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<td>75</td>
<td>775 ± 105</td>
<td>12</td>
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<td>Fumarase</td>
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<td>45</td>
<td>65.1</td>
<td>29</td>
<td>59.5</td>
<td>42</td>
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<td>Glutamate-oxaloacetate transaminase</td>
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<td>140 ± 2</td>
<td>80</td>
<td>143 ± 13</td>
<td>78</td>
<td>163 ± 24</td>
<td>18</td>
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<td>(b) Particulate enzymes</td>
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<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>100</td>
<td>6.5 ± 0.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>40.2 ± 3.0</td>
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<td>(c) Cytosol enzymes</td>
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<tr>
<td>Isocitrate lyase</td>
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<td>27.4</td>
<td>110</td>
<td>37.8</td>
<td>121</td>
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<td>Phosphoenolpyruvate carb-oxylase</td>
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<td>28.3 ± 3</td>
<td>103</td>
<td>35.5 ± 6.0</td>
<td>99</td>
<td>46.5 ± 14</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Fructose 1,6-diphosphatase</td>
<td>100</td>
<td>45.2</td>
<td>92</td>
<td>49.5</td>
<td>95</td>
<td>69.3</td>
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<td>Isocitrate dehydrogenase (NAD⁺-linked)</td>
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<td>50.7 ± 8.2</td>
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<td>50.5 ± 11</td>
<td>95</td>
<td>73.1 ± 9</td>
<td>2.8</td>
<td>6.3</td>
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<td>2-Oxo acid carboxy-lyase</td>
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<td>45.5</td>
<td>94</td>
<td>54.5</td>
<td>103</td>
<td>71.2</td>
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<td>Pyruvate carboxylase (ADP)</td>
<td>100</td>
<td>30.5</td>
<td>99</td>
<td>36.9</td>
<td>96</td>
<td>49.2</td>
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<td>—</td>
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<td>Malate dehydrogenase, decarboxy- lating (NAD⁺-linked)</td>
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<td>192</td>
<td>94</td>
<td>213</td>
<td>99</td>
<td>270</td>
<td>6</td>
<td>64.7</td>
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<td>Glucose 6-phosphate dehydrogenase</td>
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<td>21.6 ± 0.3</td>
<td>107</td>
<td>26.2 ± 0.25</td>
<td>98</td>
<td>34.3 ± 3.3</td>
<td>2</td>
<td>6.8</td>
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<td>6-Phosphogluconate dehydrogenase</td>
<td>100</td>
<td>22.3</td>
<td>109</td>
<td>31.5</td>
<td>104</td>
<td>41.6</td>
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ENZYMES OF INTERMEDIARY METABOLISM IN ASTASIA AND EUGLENA

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 48h or O₂ treatment for 48h did not change the distribution of these enzymes in Astasia.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Astasia</th>
<th>Euglena</th>
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<td>Citrate synthase</td>
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<td>Isocitrate lyase</td>
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<td>0.25</td>
</tr>
<tr>
<td>Malate synthase</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Aconitase</td>
<td>100</td>
<td>107</td>
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<tr>
<td>Isocitrate dehydrogenase (NAD⁺-linked)</td>
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<td>100</td>
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<table>
<thead>
<tr>
<th>Fraction</th>
<th>Astasia</th>
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<tr>
<td>Particulate</td>
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<th>Cytosol</th>
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<th>Euglena</th>
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<td>Phosphoenolpyruvate carboxylase</td>
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<td>2-Oxo acid carboxy-lyase (ADP)</td>
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<td>Glucose 6-phosphate dehydrogenase (ADP)</td>
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<td>302</td>
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<td>6-Phosphogluconate dehydrogenase</td>
<td>100</td>
<td>142</td>
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</table>
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 (P₁) of Euglena. The specific activity of this enzyme in the homogenate (S₁) was, however, much lower in Euglena (0.327 nmol·min⁻¹·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 (S₃) 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.

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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·min⁻¹·mg of protein⁻¹. — denotes that no activity was detected.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction...</th>
<th>S₁</th>
<th>S₂</th>
<th>S₃</th>
<th>P₁</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>Sp. act.</td>
<td>%</td>
<td>Sp. act.</td>
<td>%</td>
</tr>
<tr>
<td>(a) Dually located</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>100</td>
<td>92.8</td>
<td>35</td>
<td>44.2</td>
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<td>Isocitrate lyase</td>
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<td>2.52</td>
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<tr>
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<td>Aconitase</td>
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<td>35.8</td>
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<td>Isocitrate dehydrogenase (NAD⁺-linked)</td>
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<td>880</td>
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<td>Malate dehydrogenase,</td>
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<td>180</td>
<td>99</td>
<td>250</td>
<td>81</td>
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<tr>
<td>decarboxylating (NADP⁺-linked)</td>
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<tr>
<td>(b) Particulate</td>
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<tr>
<td>Succinate dehydrogenase</td>
<td>100</td>
<td>11.1</td>
<td>—</td>
<td>—</td>
<td>108</td>
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<tr>
<td>(c) Cytosol</td>
<td></td>
<td></td>
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<tr>
<td>Phosphoenolpyruvate</td>
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<td>155</td>
<td>92</td>
<td>200</td>
<td>92</td>
</tr>
<tr>
<td>carboxylase</td>
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<tr>
<td>Fructose 1,6-diphosphatase</td>
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<td>47.8</td>
<td>110</td>
<td>68.5</td>
<td>110</td>
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<td>Isocitrate dehydrogenase (NAD⁺-linked)</td>
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<td>2-Oxo acid carboxy-lyase</td>
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<td>71.9</td>
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<td>Pyruvate carboxylase (ADP)</td>
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ENZYMES OF INTERMEDIARY METABOLISM IN *ASTASIA* AND *EUGLENA* 613

![Graphs of enzyme activities](image)

**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 \( \cdot \) min\(^{-1} \cdot \) 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).
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.

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

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

Fraction no.
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-Heck, 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 & Beevers (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 leaking 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 glyoxy-
somes, which even centrifugation through a sucrose 
gradiant could not break. If this were so, however, the 
remained 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 be-
cause of much lower percentage of cell breakage. 
The method of disruption in the Braun MSK homo-
genizer 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.

I am indebted to the Medical Research Council of 
Canada for the award of a grant.

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