Changes in Density of Mitochondria and Glyoxysomes from *Neurospora crassa*: a Re-evaluation Utilizing Silica Sol Gradient Centrifugation

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The buoyant density of isolated mitochondria and glyoxysomes obtained from *Neurospora crassa* grown under different conditions was investigated by density-gradient centrifugation on Percoll. Mitochondria isolated from *N. crassa* in the exponential phase of growth with sucrose as the carbon source were more dense (1.077 g ml⁻¹) than those isolated from hyphae in the stationary phase (1.069 to 1.073 g ml⁻¹). A second population of mitochondria (density 1.059 to 1.061 g ml⁻¹) was also present in the stationary phase. When *N. crassa* was grown with acetate as the carbon source, mitochondria isolated from hyphae in the exponential phase of growth were found to be less dense (1.079 g ml⁻¹) than those present in the stationary phase (1.089 g ml⁻¹). The glyoxysomes were found to be less dense than the mitochondria and their density decreased from 1.076 to 1.055 g ml⁻¹ as the hyphae aged.

INTRODUCTION

The apparent buoyant density of mitochondria and glyoxysomes isolated from *Neurospora crassa* has been determined by centrifugation in a linear gradient of sucrose (Kobr & Vanderhaeghe, 1973; Ortega-Perez *et al.*, 1977). The density of mitochondria is affected by the growth conditions of *N. crassa*: the mitochondria appear more dense in conidia than in hyphae growing exponentially (Ortega-Perez *et al.*, 1977) and more dense in hyphae grown with acetate than in hyphae grown with sucrose (Kobr & Vanderhaeghe, 1973). The buoyant density of glyoxysomes is affected not only by the growth conditions of *N. crassa*, but also by the tonicity of the isolation medium (Kobr & Vanderhaeghe, 1973). The glyoxysomes from *N. crassa* appear more dense than the mitochondria. A similar situation has been reported for organelles isolated from castor bean endosperm and cucumber seeds (Cooper & Beevers, 1969; Köller *et al.*, 1979), whereas glyoxysomes are less dense than mitochondria isolated from *Coprinus lagopus* (O'Sullivan & Casselton, 1973). Furthermore, the situation is not clear for organelles isolated from *Saccharomyces cerevisiae* (Szabo & Avers, 1969; Parish, 1975) or from *Candida tropicalis* (Osumi *et al.*, 1975; Kawamoto *et al.*, 1977). These contradictory results could be due either to the experimental conditions or to the lipid and/or protein composition of the glyoxysomes, which could change according to the organisms and the growth conditions.

Since the membrane of glyoxysomes and the outer membrane of mitochondria are permeable to sucrose, this could lead to an osmotically induced change of volume and of organelle density during centrifugation. Accordingly, the buoyant density of organelles measured in sucrose gradients may not represent the real density. Recently, Percoll, a colloidal solution of silica coated with polyvinylpyrrolidone, has been made commercially available for density-gradient centrifugation. Percoll combines low viscosity, low osmolarity and non-toxicity (Pharmacia, 1980). It does not adsorb to or penetrate membranes; thus, particles band at densities more characteristic of the natural values. Discontinuous Percoll gradients can be used successfully to separate mitochondria from other plant organelles (Jackson *et al.*, 1979).
The present work describes a re-evaluation of the effect of the growth conditions of *N. crassa* on the buoyant density of mitochondria and glyoxysomes, measured by centrifugation in a Percoll density gradient.

## METHODS

### Organism and growth conditions.

*Neurospora crassa*, wild type, strain STA4 (FGSC 262) was kindly supplied by the Fungal Genetics Stock Center (Humboldt State University, Arcata, Calif., U.S.A.).

The growth medium was derived from the Neurospora minimal medium produced by Difco, with either 58 mM-sucrose or 133 mM-acetate plus 6 mM-sucrose as the carbon and energy sources. To grow hyphae, 2 l Erlenmeyer flasks containing 500 ml of the liquid medium (pH 5.7) were inoculated with 10 ml of a suspension of conidia (*A*₄₆₀ 2.5 to 3.0 for cultures grown with sucrose, 5.0 to 6.0 for cultures grown with acetate). Cultures were grown at 30°C on a rotary shaker (120 rotations min⁻¹).

When *N. crassa* was grown with 58 mM-sucrose, hyphae were harvested after 16 h, in the early exponential phase of growth (15 to 6 g wet wt (1 culture)⁻¹ or after 24 h, in the late exponential phase of growth (13 to 15 g l⁻¹). The cells reached the stationary phase after 48 h (22 to 24 g l⁻¹) and finally a stage of differentiation after 96 h (25 to 27 g l⁻¹), with a massive production of conidia. The pH of the growth medium decreased to 3-4.

### Isolation of mitochondria.

Mycelia were harvested by vacuum filtration through filter paper and washed with distilled water at room temperature. The hyphae were squeezed by hand between filter papers, weighed (wet weight) and resuspended in a minimal volume of isolation medium. The suspension was diluted in washing medium and the mitochondria were recovered by centrifugation at 48000 g for 10 min. The supernatant was further centrifuged at 40000 g for 5 min. The resulting pellet was carefully resuspended in washing medium (0.35 M-sucrose, 10 mM-N-(trishydroxymethyl)methyl-2-aminoethanesulphonic acid (TES buffer), pH 7.0, 1 mM-EGTA and 0.3% (w/v) bovine serum albumin). All subsequent operations were carried out at 4°C.

### Purification of mitochondria and glyoxysomes by centrifugation on Percoll density gradients.

A 1 ml portion of the washed mitochondrial pellet (7 to 17 mg protein) was mixed with 40 ml of a solution containing 25% (v/v) Percoll (density 1.06 g ml⁻¹), 0.25 M-sucrose and 7.5 mM-TES buffer, adjusted to pH 7.0. This was produced by mixing 30 ml of the washing medium with 10 ml of the commercially available Percoll solution (Pharmacia). The centrifugation was carried out at 48000 g for 1 h in a 50 ml tube in a Sorvall RC2-B centrifuge, using a SS-34 rotor, to generate the gradient and separate the organelles.

After centrifugation, the gradient was collected by pumping from the bottom of the tube and was monitored through an ultraviolet analyser (Uvikord II; LKB Produkter, Sweden). The distribution of organelles in the gradient could be determined according to the absorbance of protein at 280 nm, although Percoll itself was observed to scatter light in the ultraviolet region (Fig. 1). Fractions of 0.4 to 0.7 ml were collected and their density was calculated from their refractive index measured at room temperature. The shape of the density gradient was clearly sigmoidal (Fig. 1). The activities of several marker enzymes of mitochondria and glyoxysomes were measured in the different fractions without further treatment.

### Measurements of enzyme activities.

NAD⁺-isocitrate dehydrogenase (EC 1.1.1.41) was assayed in 50 mM-TES buffer, pH 7.4, which contained 0.3 M-sucrose, 5 mM-DL-isocitrate, 0.25 mM-AMP, 1 mM-MgSO₄, 3 mM-KCN, 1 mM-NAD⁺ and 0.02% (w/v) Triton X-100. The reaction was started by the addition of NAD⁺ and was measured by following the increase in A₄₅₀. NAD⁺-isocitrate dehydrogenase (EC 1.1.1.42) was measured as for the NAD⁺-linked enzyme, except that 1 mM-NAD⁺ was added instead of NAD⁺. The activity of isocitrate lyase (EC 4.1.3.1) was measured in a reaction medium containing 50 mM-TES buffer, pH 7.4, 0.3 M-sucrose, 2.5 mM-MgSO₄, 10 mM-DL-isocitrate and 3 mM-phenylhydrazine. The formation of the glyoxylate--
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Fig. 1. Refractive index (■) and distribution of protein (A360 ○) after density-gradient centrifugation on Percoll of a mitochondrial pellet from *N. crassa*. The culture was grown on acetate for 66 h. A preparation containing 13.5 mg protein was applied to the gradient and 0.6 ml fractions were collected. The distribution of protein is expressed in arbitrary units, according to the chart of the recorder connected to the ultraviolet analyser.

phenylhydrazone complex was followed at 324 nm (ε 17000 M⁻¹ cm⁻¹). The malate dehydrogenase (EC 1.1.1.37) activity was determined by following the oxidation of NADH at 340 nm in a reaction medium containing 90 mM-Tris/HCl buffer, pH 7.5, 2 mM-KCN, 0.02% Triton X-100, 2.0 mM-oxaloacetate and 0.15 mM-NADH. Succinate dehydrogenase (EC 1.3.99.1) was activated by preincubation of the mitochondria at room temperature for 5 min in a mixture of 0.35 M-sucrose, 1.7 mM-MgCl₂, 17 mM-ATP and 17 mM-phosphate buffer, pH 7.2. The enzyme activity was then measured in a reaction medium containing 30 mM-phosphate buffer, pH 7.4, 0.1% bovine serum albumin, 0.01% Triton X-100, 2 mM-KCN, 0.1 mM-EDTA, 1.4 mM-phenazine methosulphate and 0.044 mM-2,6-dichlorophenolindophenol (DCPIP). The reaction was started by the addition of 20 mM-succinate and the decrease in A₆0₀ was followed (ε 21000 M⁻¹ cm⁻¹). The activity of citrate synthase (EC 4.1.3.7) was measured in a medium containing 90 mM-Tris/HCl buffer, pH 8.0, 0.02% Triton X-100, 2.5 mM-oxaloacetate, 0.2 mM-acetyl-CoA and 0.1 mM-5,5'-dithiobis(2-nitrobenzoic acid). The increase in A₄₁₂ was followed (ε 15 700 M⁻¹ cm⁻¹).

Protein estimation. Protein was measured by the method of Lowry with a standard of bovine serum albumin.

**RESULTS**

*N. crassa* grown with sucrose

Mitochondria isolated from 16 h cultures, in the early exponential phase of growth, appeared as a homogeneous population after centrifugation in a Percoll gradient (Fig. 2). NAD⁺-isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase were found in the same fractions recovered from the gradient, at a mean buoyant density of 1.077 g ml⁻¹. A small amount of all three enzymes was also found at the top of the gradient and may represent soluble enzymes derived from broken mitochondria. A similar distribution of enzymes was observed for mitochondria isolated from 24 h cultures, in the late exponential phase of growth, showing that the buoyant density of mitochondria from *N. crassa* did not change during the exponential phase of growth (Table 1a).

In the stationary phase of growth (48 h cultures), the buoyant density of isolated mitochondria was found to decrease to 1.073 g ml⁻¹ and a second population of mitochondria was found with a lower density of 1.061 g ml⁻¹ (Table 1a).

Two populations of mitochondria, with buoyant densities of 1.069 and 1.059 g ml⁻¹, were obtained from hyphae grown for 96 h, when differentiation of the conidia was apparent.
Fig. 2. Percoll density gradient centrifugation of a mitochondrial pellet isolated from *N. crassa* grown with sucrose. Enzyme activities are expressed as units per fraction. A unit is defined as 10 nmol NAD$^+$ reduced min$^{-1}$ for isocitrate dehydrogenase (○), 10 nmol DCPIP reduced min$^{-1}$ for succinate dehydrogenase (■), and 100 nmol NADH oxidized min$^{-1}$ for malate dehydrogenase (●). A preparation containing 7.3 mg protein, isolated from a 16 h culture, was applied to the gradient and fractions of 0.45 ml were collected.

### Table 1. Relation between the growth conditions of *N. crassa* and the buoyant density of its organelles

Mitochondria and glyoxysomes were isolated and purified by centrifugation on Percoll density gradients as described in Methods. The buoyant densities were determined according to the refractive index of the peak fractions containing marker enzymes of the organelles.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth period (h)</th>
<th>Mitochondria (major peak)</th>
<th>Mitochondria (minor peak)</th>
<th>Glyoxysomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>(a)</em> Sucrose</td>
<td>16</td>
<td>1.077</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.077</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.073</td>
<td>1.061</td>
<td>1.067</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>1.069</td>
<td>1.059</td>
<td>1.076</td>
</tr>
<tr>
<td><em>(b)</em> Acetate</td>
<td>26</td>
<td>1.078</td>
<td>1.062</td>
<td>1.072</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.078</td>
<td>—</td>
<td>1.072</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>1.089</td>
<td>—</td>
<td>1.055</td>
</tr>
</tbody>
</table>

(Table 1a). Both contained high activities of NAD$^+$-isocitrate dehydrogenase and succinate dehydrogenase, but a much lower activity of malate dehydrogenase, as compared with mitochondria from the exponential or the stationary phase of growth.

The activity of isocitrate lyase in the particulate fraction was derepressed in the stage of differentiation. Although the distributions of isocitrate lyase and of mitochondrial enzymes overlapped extensively, the maximal activities were not found in the same fractions and the glyoxysomes were somewhat less dense than the mitochondria (Table 1a).

*N. crassa* grown with acetate

When hyphae were harvested in the early exponential phase of growth (26 h), the mean buoyant density of isolated mitochondria was found to be 1.078 g ml$^{-1}$ (Table 1b). This
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Fig. 3. Percoll density gradient centrifugation of a mitochondrial pellet isolated from \textit{N. crassa} grown with acetate. Enzyme activities are expressed as units per fraction. A unit is defined as 20 nmol NAD$^+$ reduced min$^{-1}$ for isocitrate dehydrogenase (O), 100 nmol DCPIP reduced min$^{-1}$ for succinate dehydrogenase (O), 1 mmol NADH oxidized min$^{-1}$ for malate dehydrogenase (O), and 5 nmol glyoxylate produced min$^{-1}$ for isocitrate lyase (A) (note differences in units from Fig. 2). A preparation containing 15.0 mg protein, isolated from a 48 h culture, was applied to the gradient and fractions of 0.7 ml were collected.

The distribution of the mitochondrial marker enzymes and of isocitrate lyase was not identical, although an extensive overlapping was observed: glyoxysomes appeared slightly less dense than mitochondria (Table 1b). The distribution of the mitochondrial marker enzymes was similar in preparations obtained from \textit{N. crassa} harvested after 26 h or 48 h of growth with acetate (Table 1b). The density of glyoxysomes decreased from 1.076 to 1.072 g ml$^{-1}$ during the exponential phase of growth and a malate dehydrogenase activity was clearly associated with them (Fig. 3).

In the stationary phase of growth with acetate (66 h), the buoyant density of isolated mitochondria increased to 1.089 g ml$^{-1}$, whereas the density of glyoxysomes decreased to 1.055 g ml$^{-1}$ (Table 1b).

It should be pointed out that for both exponential and stationary phases of growth, the activity of isocitrate lyase was distributed in three peaks. In the exponential phase of growth, the main peak of isocitrate lyase could correspond to ‘heavy’ glyoxysomes, scarcely less dense than mitochondria and containing malate dehydrogenase. In the stationary phase, the ‘light’ population of glyoxysomes was predominant and only a low malate dehydrogenase activity was found in these organelles. For all phases of growth in the presence of acetate, the activities of citrate synthase and NADP$^+$-isocitrate dehydrogenase were clearly associated with mitochondria, but not with glyoxysomes. Kobr \textit{et al.} (1965) reported that the activity of NAD$^+$-isocitrate dehydrogenase was somewhat enhanced by growing \textit{N. crassa} with acetate. The results presented here show that the activities of malate dehydrogenase and
succinate dehydrogenase associated with the mitochondrial fraction are also markedly increased (compare Figs 2 and 3 – note differences in units).

**DISCUSSION**

The apparent buoyant density of mitochondria from *N. crassa*, determined by centrifugation on a linear sucrose gradient, ranged from 1.18 to 1.21 g ml\(^{-1}\) depending on the growth conditions (Kobr & Vanderhaeghe, 1973; Ortega-Perez et al., 1977). Unlike sucrose, Percoll does not penetrate biological membranes and does not exert large osmotic effects. Subcellular organelles therefore band at densities lower than on sucrose gradients and densities from 1.04 to 1.10 g ml\(^{-1}\) have been recorded for different types of mitochondria (Pharmacia, 1980). This work shows that on Percoll gradients *N. crassa* mitochondria band at densities from 1.06 to 1.09 g ml\(^{-1}\), depending on the growth conditions. These densities are lower than those obtained with sucrose gradients and are probably much closer to the in vivo values.

When *N. crassa* was harvested in the exponential phase of growth, the density of isolated mitochondria was similar for sucrose- and acetate-grown cultures. In contrast, in the stationary phase the mitochondria became less dense when the hyphae were grown with sucrose and more dense when grown with acetate. Furthermore, in sucrose-grown cultures, a second, less dense, mitochondrial population appeared before the formation of conidia. This is not in agreement with results obtained by centrifugation on sucrose density gradients, where mitochondria from conidia appeared more dense than mitochondria from growing hyphae (Ortega-Perez et al., 1977).

Changes in mitochondrial density should reflect changes in the lipid and/or protein composition of the organelle. Little is known concerning the changes in the ratio of protein to lipid in mitochondria during the growth and the differentiation of *N. crassa*. However, for the *chol-I* mutant of *N. crassa*, the density of mitochondria is determined partially by the concentration of choline in the culture medium. At low choline concentrations, mitochondria are more dense than at high choline concentrations (Luck, 1965). Furthermore, changes occur in the synthesis of mitochondrial phospholipids during the germination of conidia and the early exponential period of growth of wild-type *N. crassa* (Beck & Greenawalt, 1976).

Depending on the growth conditions, the apparent density of glyoxysomes from *N. crassa* was found by Kobr & Vanderhaeghe (1973) to be from 1.20 to 1.22 g ml\(^{-1}\). In previous studies, the glyoxysomes thus appeared to be more dense than mitochondria. Furthermore, their density is affected by the tonicity of the surrounding medium. When glyoxysomes are suspended in 1.45 M-sucrose, only one population is found at 1.22 g ml\(^{-1}\). A second band is found at 1.16 or 1.14 g ml\(^{-1}\) when glyoxysomes are suspended in 1.16 or 0.93 M-sucrose, respectively (Kobr & Vanderhaeghe, 1973). Since the sucrose concentration was extremely high in these experiments, the determination of the organelle density was probably erroneous. From the results presented in this paper, the glyoxysomes, suspended in 0.35 M-sucrose and centrifuged in a Percoll density gradient, appeared less dense than mitochondria, with a density range from 1.055 to 1.076 g ml\(^{-1}\), depending on the growth conditions. For the reasons outlined above, these values should be reasonably close to the in vivo densities of glyoxysomes.

In *N. crassa*, citrate synthase, NAD\(^+\)- and NADP\(^+\)-isocitrate dehydrogenases, and succinate dehydrogenase are present only in the mitochondria, whereas isocitrate lyase is found in glyoxysomes, but not in mitochondria. Our results show that a malate dehydrogenase activity is associated with the 'heavy' glyoxysomes isolated from *N. crassa* growing exponentially on acetate. In the stationary phase however, no malate dehydrogenase could be found in the 'light' glyoxysomes. This observation could explain the contradictory reports concerning the presence (Kobr et al., 1969) or the absence (Kobr & Vanderhaeghe, 1973) of malate dehydrogenase in glyoxysomes from *N. crassa*, since the growth conditions...
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employed in the two studies were different. Like the glyoxysomes from *Tetrahymena pyriformis* (Hogg, 1969) or from *Candida tropicalis* (Fukui & Tanaka, 1979), the glyoxysomes from *N. crassa* have to import a substrate for isocitrate lyase and to export succinate, and possibly also malate, for further oxidation in the mitochondria. The synthesis of precursors for gluconeogenesis would therefore be regulated by a cooperation between glyoxysomes and mitochondria.

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