Short Communication

Use of Dextran-40 Gradients for Separation of Pea Cotyledon Mitochondria into Different Fractions

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ABSTRACT

A crude pea (Pisum sativum L. var. Homesteader) mitochondrial preparation was divided into two equal parts. One part was layered on a Dextran-40 step gradient, and the other on a sucrose step gradient, and they were centrifuged to obtain different bands of particles. The densities at which the particles banded and the mitochondrial respiratory activities of the particles were determined. Dextran-40 density gradient centrifugation resulted in a better separation of mitochondrial populations than did sucrose density gradient centrifugation. Separation by sucrose density gradient centrifugation may not be according to the true densities of the particles. On the other hand, the use of gradients of Dextran-40, a solute of low osmotic potential, facilitated separation of particles according to their true densities. Such mitochondria showed better respiratory control ratio and ADP:O values, than those isolated by sucrose density gradient centrifugation.

Separations of subcellular particles have been greatly enhanced by the use of isopycnic centrifugation techniques. The density of a subcellular particle in a solution is affected primarily by the osmotic characteristics of the solutes and the permeability towards the solutes of the membrane(s) enveloping the particle. If the osmoticant can penetrate to the interior of the particle, then the "osmotant space" within the particle will also influence the separation. Sucrose density gradients are widely used for separations of subcellular particles.

In fully developed mitochondria, only the outer membrane is permeable to sucrose. Thus the equilibrium density (1.18–1.20 g/ml [2, 4]) of these particles is determined primarily by the density of the matrix of mitochondria. However, mitochondria at early stages of development have only partially developed membrane barriers to sucrose penetration. They exhibit a variety of sucrose equilibrium densities, depending on their stage of development (5–7).

It has been shown by various workers that equilibrium densities for subcellular particles can be varied by use of solutes of low osmotic potentials (2). Separation based on such solutes will depend on the true density of the particles, for the large molecules of solute do not penetrate mitochondrial membranes. Such separations should eliminate the osmotant space factor in the separations. In the present study, we have separated different populations of pea cotyledon mitochondria by use of Dextran-40.

MATERIALS AND METHODS

A homogenate was prepared from 50 g of 4-day-old germinating pea seeds (Pisum sativum L. var. Homesteader) by gently grinding the cotyledons with a mortar and pestle for 3 min in 100 ml of the following grinding buffer: 0.5 mM mannitol, 0.005 M EDTA, 0.5% bovine serum albumin, 0.05% cysteine, 0.05 M TES, pH 7.4 at 0 C. The homogenate was centrifuged (2500g for 7 min, followed by 40,000g for 5 min [6]) to obtain a crude mitochondrial pellet. The crude mitochondrial pellet (1.8–2 mg of protein) was suspended in 0.75 ml of the following suspending medium: 0.3 M mannitol, 0.004 M MgCl₂, and 0.05 M TES, pH 7.2, at 0 C. The suspension was divided into two equal parts. One part was layered on a 5–10–20–25–30% (w/w) Dextran-40 step gradient and the other on a 38–44.83–48–60% (w/w) sucrose step gradient. They were centrifuged for 3.5 hr at 144,000g in a swing-bucket rotor in a Model L2-65 B Spinco ultracentrifuge. The Dextran-40 solutions were all made in 0.25 mol sucrose to 0.05 M TES buffer, pH 7.4, at 0 C. All steps were carried out at 0 to 4 C.

RESULTS AND DISCUSSION

The crude mitochondrial pellet was isolated from cotyledons of 4-day-old germinating pea seeds. We have previously found that on the 4th day of germination, most of the mitochondria become fully developed and band at the 44.83% sucrose step (sucrose equilibrium density of 1.205); a lesser amount of mitochondria band at 38% sucrose step (sucrose equilibrium density of 1.1739). All the mitochondria that band at 44.83% sucrose step have a uniform density under these conditions and might be thought to represent a single population. However, a uniform sucrose equilibrium density does not necessarily reflect a uniform matrix density. A uniform sucrose equilibrium density for different populations can also result from the physical characteristics of mitochondria such as the extent of membrane(s) development and the amount of "sucrose space" (6) within the particles. If this is so, mitochondria with varying amounts of sucrose space, when isolated by sucrose density gradient centrifugation, could take up different amounts of
sucrose, become heavier, and band at the same sucrose equilibrium density as that of fully developed mitochondria.

It has been demonstrated that the equilibrium densities for subcellular particles can be varied by use of solutes of low osmotic potential (4) such as Dextran-40. The densities at which the particles settle in the Dextran-40 gradient should be true densities, because the large Dextran-40 molecules do not penetrate mitochondrial membranes. Dextran-40 was, therefore, successfully used to separate different populations of mitochondria that would normally separate into only two populations, when sucrose was used instead of Dextran-40 (Tables I–IV).

At the end of the centrifugation, it was found that mitochondrial activity was present in all five steps of the Dextran-40 gradient (Tables I and II) and the top two steps (38 and 44.83%) of the sucrose gradient (Tables III and IV). The highest amount of protein was present in the 10% step of the gradient.

### Table I. Mitochondrial Activities (Measured as Ability to Oxidize Succinate) in Different Dextran-40 Step Density Gradient Fractions from Cotyledons of 4-day-old Germinating Pea Seeds

The crude mitochondrial suspension was divided into two equal parts, one used for dextran and one for sucrose (Table II) step density gradient centrifugations. The concentrations of the chemicals in the assay mixture were as follows: 0.3 mM mannitol, 4 mM MgCl₂, 5 mM K₂HPO₄, 50 mM TES buffer, pH 7.4, at 25°C, 8 mM succinate, 0.75 mg of bovine serum albumin/mL. ADP (0.3 μmole) was added at least twice to get more than one cycle for the determination of respiratory control and ADP:O ratios. The reaction was run at 25°C.

### Table II. Mitochondrial Activities (Measured as Ability to Oxidize α-Oxoglutarate) in Different Dextran-40 Step Density Gradient Fractions from Cotyledons of 4-day-old Germinating Pea Seeds

### Table III. Mitochondrial Activities (Measured as Ability to Oxidize Succinate) in Different Sucrose Step Density Gradient Fractions from Cotyledons of 4-day-old Germinating Pea Seeds

Experimental conditions were the same as in Table I.

<table>
<thead>
<tr>
<th>Step</th>
<th>Sucrose</th>
<th>Step Density</th>
<th>Calculated Osmotic Potential</th>
<th>Rate of O₂ Uptake</th>
<th>ADP:O</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>g/ml</td>
<td>atm</td>
<td>mmoles O₂/min/mg protein</td>
<td>ratio</td>
<td>mg/g step</td>
</tr>
<tr>
<td>5</td>
<td>1.023</td>
<td>0.028</td>
<td>6.582</td>
<td>27.8</td>
<td>7.5</td>
<td>3.7</td>
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<td>0.057</td>
<td>6.611</td>
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<td>6.668</td>
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<td>3.5</td>
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<td>0.171</td>
<td>6.725</td>
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<tr>
<td>Crude pellet</td>
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<td></td>
<td></td>
<td>19.6</td>
<td>5.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

### Table IV. Mitochondrial Activities (Measured as Ability to Oxidize α-Oxoglutarate) in Different Sucrose Step Density Gradient Fractions from Cotyledons of 4-day-old Germinating Pea Seeds

Experimental conditions were the same as in Table III.

<table>
<thead>
<tr>
<th>Step</th>
<th>Dextran</th>
<th>Step Density</th>
<th>Calculated Osmotic Potential</th>
<th>Rate of O₂ Uptake</th>
<th>ADP:O</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>g/ml</td>
<td>atm</td>
<td>mmoles O₂/min/mg protein</td>
<td>ratio</td>
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<td>44.83</td>
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<td>48.0</td>
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<td>31.96</td>
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<tr>
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<td></td>
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<td>19.6</td>
<td>5.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Dextran-40 density gradient and the 44.83% step of the sucrose density gradient. (The data in different tables are representative of several runs and, the variation between the runs was minimal.)

Particles that banded in the 10% Dextran-40 step showed the highest RCR and ADP:O values with either succinate or α-oxoglutarate as substrates (Tables I and II). Those banded in the 5 and 20% Dextran-40 step showed very little or no difference between themselves with regard to their RCR and ADP:O ratios with either substrate. Finally, those that banded in the 25 and 30% Dextran-40 steps did not respond to the addition of ADP in the presence of either substrate. Tables I and II show that the osmopotentials of all the Dextran-40 steps are very similar, in contrast to those for sucrose steps (Tables III and IV). The osmotic potentials were determined according to Van't Hoff's equation. Mitochondria that had been subjected to different osmotic potentials, upon retesting, were found not to have lost their respiratory activities. (Retesting was carried out after dilution of the osmoticant, centrifugation, and resuspension of the resulting pellet in 0.3 mM mannitol, 0.004 mM MgCl₂, and 0.5 mM TES, pH 7.2, at 0°C.)

The RCR and ADP:O values of particles from the 5 and 20% steps were also determined. The results will be reported in a subsequent study.

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*Abbreviations: RCR: respiratory control ratio (rate of oxygen utilization when ADP is present: rate of oxygen utilization when ADP is limiting); ADP:O: adenosine diphosphate–oxygen (μmoles of ADP esterified: μatoms of oxygen consumed).
20% Dextran-40 steps, with succinate as substrate, do not differ greatly from those of the mitochondria of the crude pellet (Table I). When α-ketoglutarate is used as substrate, RCR values of the 5 and 20% Dextran-40 steps remained unchanged as compared to those from the crude pellet while the ADP:O values improved (Table II). These results indicate that metabolic characteristics of mitochondria have not been drastically altered by Dextran-40. The RCR, particularly, of particles from the 10% Dextran-40 step are better than those of the crude pellet (Tables I and II). Consequently, the differences in the respiratory characteristics of the mitochondria in the various bands may reflect intrinsic differences in their mitochondrial populations.

The contribution by Dextran-40 to the osmotic pressures of the gradient steps varies from 0.028 to 0.171 atmosphere, assuming a linear relationship of concentration and osmotic pressure (Tables I and II). However, a linear relation may not exist, for the amount of water free to act as a solvent will be decreased because of the water binding properties of the hydrophilic colloid. (The data supplied by the Pharmacia Company for Ficoll show that linearity of osmotic pressure vs. concentration ceases to exist after about 1% w/w.)

The slight effect of Dextran-40 on the osmotic pressure (Tables I and II) might alter the position in which the mitochondria might be expected to band. Since the osmotic potential of 5% Dextran-40 alone is negligible (0.028 atm [Tables I and II]), it would be anticipated that mitochondria, if uniform, would band at this step (3). But, after the density gradient centrifugation, several bands with different densities were observed (Tables I and II). This may be interpreted as being a result of differences in matrix densities of the mitochondria themselves. Alternatively, the wide spectrum of density and the separation achieved may result from surface interaction of mitochondria and Dextran. There may be different surface areas and/or surface properties for different populations. Others have shown that subcellular particles can be partitioned between two immiscible solutions of polymers, and this partition was attributed to surface effects (1). Whatever the reason may be, it appears to be possible to separate mitochondria with different densities and metabolic characteristics.

Two different populations of mitochondria were determined when the crude pellet was subjected to sucrose equilibrium density gradient centrifugation (Tables III and IV). With succinate as substrate, the rate of oxygen uptake in both state 3 and state 4 of mitochondria in the 44.83% sucrose step showed a large increase over that of the crude pellet (Table III), indicating higher mitochondrial purity (6). Table III also shows that mitochondria in the 44.83% sucrose step have a somewhat higher RCR value than that of the crude pellet. This may be a result of a greater increase of respiration in state 3 than in state 4 after sucrose density gradient centrifugation. A similar pattern of results was obtained when α-oxoglutarate was used as substrate (Table IV).

In general, mitochondrial activity in the 38% sucrose step (with either substrate) remained more or less unchanged as compared to that from the crude pellet except for the RCR (with succinate as substrate); the rate of state 3 oxygen uptake (with either substrate) declined after sucrose density gradient centrifugation.

Separations indicate that the mitochondria that banded in the 38 and 44.83% sucrose steps (Tables III and IV) may consist of more than two populations (Tables I and II). The oxygen utilization in state 3 by different populations of mitochondria isolated by Dextran-40 density gradient centrifugation is lower than that of mitochondria isolated by sucrose density gradient centrifugation, but Dextran-40 can apparently be used more effectively to isolate different populations of mitochondria. Such mitochondria were shown to have better RCR and ADP:O values as compared to those isolated by sucrose density gradient centrifugation.

Acknowledgment—Our thanks are due to Dr. Park Nobel for an interesting discussion.

LITERATURE CITED