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Method to Obtain a Chlorophyll-free Preparation of Intact Mitochondria from Spinach Leaves[1]

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ABSTRACT

Mitochondria from green leaves of spinach have been prepared using a three-step procedure involving differential centrifugation, partition in an aqueous dextran polyethylene glycol two-phase system and Percoll gradient centrifugation. The mitochondrial fractions after the different steps of purification were compared. The final mitochondrial preparation was totally free from chloroplast material measured as chlorophyll content. The enrichment of mitochondria in relation to peroxisomes and microsomes was approximately 12 and 33 times, respectively, based on NAD:isocitrate dehydrogenase activity, glycolate oxidase activity, and NADPH:cytochrome *c* oxidoreductase activity. The apparent intactness of the inner and the outer mitochondrial membranes was higher than 90% as measured by latency of enzyme activities. The mitochondria showed high respiratory rates with respiratory control and the ADP/O ratios approached the theoretical limits.

The discovery of the process of photorespiration has increased the interest in mitochondria from photosynthetic tissue. By differential centrifugation functional mitochondrial preparations can be obtained (3, 16). These preparations contain a great deal of contaminating material, mainly thylakoid membrane fragments; the protein to Chl ratio is approximately 15 (3, 6). The heavy contamination makes the preparations less suited for studies of molecular composition and enzyme distribution. We have developed a preparation procedure combining differential centrifugation and partition in an aqueous dextran-PEG two-phase system (referred to as "phase partition") (5) giving functional mitochondrial preparations with protein to Chl ratios of about 100. Recently about the same degree of purification has been reported using differential centrifugation and Percoll density gradient centrifugation (6, 7).

In this study we have, by using Percoll density gradient centrifugation, further purified the preparation obtained by differential centrifugation and phase partition. We have thereby been able to remove chlorophyll completely from the mitochondrial preparation while retaining function and intactness of the mitochondria.

MATERIALS AND METHODS

Leaf Material and Reagents. Spinach (*Spinacia oleracea L.* var. Viking II) was grown at 18 C in nutrient solution (14) with doubled Fe concentration, under artificial light with a light period of 12 h. Fully expanded leaves with the mid-vein removed were used in the preparations. Dextran and Percoll were obtained from Pharmacia (Uppsala, Sweden) and PEG from Union Carbide (New York). Solutions were prepared and pH was measured at room temperature.

Preparation of Mitochondria. The first steps in the preparation were performed essentially as described earlier (5). The preparation medium was composed of: 0.3 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 25 mM Hepes (pH 7.8), 0.1% (w/v) BSA (defatted), 4 mM cysteine, and 0.6% (w/v) insoluble PVP (acid washed). After a short homogenization followed by filtration, chloroplasts and cell debris were sedimented by centrifugation at 5,000*g* for 2 min. The mitochondria in the supernate were then spun down at 20,000*g* for 2.5 min. The crude mitochondrial pellet was suspended in top phase (phase system, see below), bottom phase was added, the phases were mixed and then separated by a short centrifugation. The top phase containing mostly thylakoid membrane fragments was withdrawn and the bottom phase was repartitioned once or twice with fresh top phase. The extracted bottom phase and the interface were diluted eight times with preparation medium without cysteine and PVP and centrifuged at 5,000*g* for 2 min. Finally, the mitochondria were collected from the supernate by centrifugation at 11,000*g* for 10 min. The phase system contained 5.8% (w/w) dextran 500 batch 2836 (mol wt 500,000), 5.8% (w/w) PEG 4,000 (Mn 3,000–3,700), 5 mmol/kg K-phosphate (pH 7.8), 2 mmol/kg KCl and 0.3 mol/kg sucrose. Differences in mol wt distribution between different dextran batches were found to have an influence on the critical point, the minimum polymer concentration for obtaining two phases (9). This strongly affects the partition of material in the phase systems. The present system containing 5.8% (w/w) dextran 500 batch 2836 and 5.8% (w/w) PEG 4,000 and the system used earlier (5) containing 6.1% (w/w) dextran 500 batch 5996 and 6.1% (w/w) PEG 4,000 both have polymer concentrations 0.5% (w/w) higher than the concentrations at the critical point. Both phase systems give similar separation of mitochondria and thylakoid membranes. The critical point was determined at 4 C. An eight gram phase system with 5.8% (w/w) polymer concentration was made, 0.15 ml 0.3 M sucrose was added, the system was thoroughly mixed, and then centrifuged for phase separation. Successive sucrose additions were then made until no phase separation occurred. This gives the concentration of polymers at the critical point within 0.1% (w/w).

The pellet obtained after phase partition was suspended in 0.25 M sucrose, 1 mM KH_2PO_4, 10 mM Hepes (pH 7.2) and 0.1% (w/v) BSA (defatted). About 2 ml of this suspension was layered on top of a discontinuous Percoll gradient. The gradient was prepared in 12-ml cellulose nitrate tubes by layering 2 ml 60% (v/v) Percoll, 3 ml 27% (v/v) Percoll, and 2 ml 21% (v/v) Percoll. In addition to Percoll all layers contained 0.25 M sucrose, 1 mM KH_2PO_4, 10 mM Hepes (pH 7.2), and 0.1% (w/v) BSA (defatted). The gradient was centrifuged at 10,000 rpm ($9{,}000g_{max}$) for 30 min in a Beckman 40 rotor (fixed angle) using a Beckman L2-65K ultracentrifuge. After centrifugation the mitochondrial band, on the interface between the 27% (v/v) and 60% (v/v) Percoll layers, was taken out by

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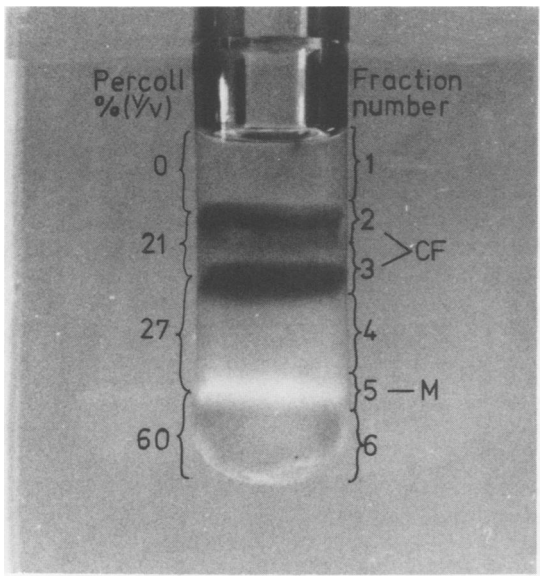

FIG. 1. Discontinuous Percoll density gradient centrifugation of the mitochondrial fraction obtained by differential centrifugation and phase partition. The fractions indicated refer to Figure 2. M: mitochondria; CF: chloroplast fragments.

Table I. *Respiration*

Respiration was measured after two different steps in the preparation, phase partition and Percoll gradient centrifugation. Values for "state 3" and "state 4" refer to nmol O_2 consumed min^{-1} mg^{-1} protein after the second and third ADP addition. Mean values and standard deviations are based on six preparations.

	NADH	Glycine	Succinate
Phase partition			
State 3	110 ± 36	59 ± 15	81 ± 16
State 4	79 ± 33	36 ± 16	63 ± 17
RC	1.5 ± 0.2	1.9 ± 0.6	1.3 ± 0.1
ADP/O	1.3 ± 0.5	2.3 ± 0.6	1.4 ± 0.4
Percoll gradient			
State 3	146 ± 42	96 ± 30	115 ± 31
State 4	107 ± 23	47 ± 20	85 ± 17
RC	1.4 ± 0.2	2.2 ± 0.6	1.4 ± 0.2
ADP/O	1.5 ± 0.5	2.5 ± 0.4	1.6 ± 0.4

puncturing the tube from the side with an injection needle. The volume of the mitochondrial fraction was about 1 ml. Other fractions from the gradient were taken out from the top of the tube.

Mitochondrial Assays. Respiration was measured at 25 C with a Hansatech O_2 electrode (Norfolk, England) in 0.4 ml respiration medium (0.3 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 0.1% (w/v) BSA (defatted), 10 mM K-phosphate (pH 7.2) with 0.05 to 0.3 mg mitochondrial protein). Substrate concentrations were 10 mM for succinate and glycine and 1 mM for NADH. The electrode was calibrated against air-saturated water, whose O_2 concentration was taken as 250 μM. ADP/O and RC ratios were measured as described by Estabrook (4).

IDH[2] activity was measured as described previously (5) in 0.3 M sucrose, 4 mM trisodium-threo-D_s-L_s-isocitrate, 1 mM $MnSO_4$, 0.5 mM KCN, 50 mM Hepes (pH 7.6), 0.6 mM NAD, and 0.02% (w/v) Triton X-100. Cyt *c* oxidase activity (EC 1.9.3.1) was measured in 10 mM K-phosphate (pH 7.0) and 50 μM reduced Cyt *c* using the method of Wharton *et al* (15). Succinate:Cyt *c* oxidoreductase activity (EC 1.3.99.1) was measured in 50 μM Cyt *c*, 1 mM KCN, 200 μM ATP, 5 mM K-phosphate (pH 7.2) and 10 mM succinate. The apparent intactness of the inner membrane was estimated from the IDH activity in isotonic medium with and without Triton X-100. The apparent intactness of the outer mitochondrial membrane was estimated from the succinate:Cyt *c* oxidoreductase activity in isotonic and hypotonic medium. Glycolate oxidase activity (EC 1.1.3.1) was measured with an O_2 electrode in respiration medium with the addition of 10 mM glycolate and 1 mM KCN. NADPH:Cyt *c* oxidoreductase activity (EC 1.6.2.4) was measured in 50 mM K-phosphate (pH 7.7), 36 μM Cyt *c*, 1 mM KCN, 0.5 μg/ml antimycin A and 0.1 mM NADPH according to Masters *et al.* (11).

The molar absorption coefficients 21.1 × 10^3 $M^{-1}cm^{-1}$ at 550 nm for Cyt *c* and 6.2 × 10^3 $M^{-1}cm^{-1}$ at 340 nm for NADH were used. Protein was determined according to the method of Lowry *et al.* (10), using BSA (defatted) as a standard protein, and Chl according to Bruinsma (1).

RESULTS AND DISCUSSION

The mitochondrial fraction prepared by differential centrifugation and phase partition was layered onto a discontinuous Percoll gradient. Figure 1 shows a picture of the gradient after centrifugation. Chloroplast material forms two green bands on top of the 21% (v/v) and the 27% (v/v) Percoll layers, whereas mitochondria form a Chl-free band on top of the 60% (v/v) Percoll layer. Fractions were collected from the gradient as indicated in Figure 1.

The fractions were assayed for mitochondria (IDH and Cyt *c* oxidase activities), broken chloroplasts (Chl content), peroxisomes (glycolate oxidase activity), microsomes (NADPH:Cyt *c* oxidoreductase activity), and protein content (Fig. 2). The distribution of Chl confirms that the mitochondrial fraction (fraction 5) is totally free from thylakoid contamination. About 65% of the mitochondria layered on the gradient is recovered in the mitochondrial fraction measured both as the IDH and Cyt *c* oxidase activities. The recovery of glycolate oxidase activity in the mitochondrial fraction is about 40%. The catalase activity was distributed similarly to the glycolate oxidase activity (results not shown). About 30% of the NADPH:Cyt *c* oxidoreductase activity layered on the gradient is found in the mitochondrial fraction. As the recovery of mitochondrial activities is about twice the recovery of glycolate oxidase and NADPH:Cyt *c* oxidoreductase, the contamination by peroxisomes and microsomes is halved in the Percoll gradient centrifugation step.

The intactness of the mitochondrial membranes based on the latencies of enzyme activities after the phase partition step was 93% ± 4% for the inner membrane and 86% ± 10% for the outer membrane. The intactness of the membranes was increased in the Percoll gradient centrifugation step to 95% ± 3% for the inner membrane and 93% ± 4% for the outer membrane. The intactness of the outer mitochondrial membrane after the phase partition step varies between preparations, probably depending on the quality of the leaves. The membrane integrity after gradient centrifugation is consistently high, indicating the removal of ruptured mitochondria on the gradient. Results of Nishimura *et al.* (12) indicate that IDH is, at least partly, a matrix enzyme as its distribution in a sucrose gradient differs from the distribution of Cyt *c* oxidase. This would lead to an overestimation of the integrity of the inner membrane using the latency of IDH activity. The latency of succinate:Cyt *c* oxidoreductase (2) is regarded as a sensitive indicator of mitochondrial integrity (13). Regardless of the accuracy of the IDH assay for inner membrane integrity our values for the succinate:Cyt *c* oxidoreductase latency show a very high integrity of the mitochondria after Percoll gradient centrifu-

[2] Abbreviation: IDH: isocitrate dehydrogenase (EC 1.1.1.41) (threo-D_s-isocitrate:NAD oxidoreductase [decarboxylating]).

Table II. *Recovery of Activities after the Different Steps of Purification*

The values are expressed as average percent of the total amount in the filtrate and are based on at least six preparations. Figures in parentheses show the maximum and minimum values obtained from different preparations. Before measurement of the IDH activity the filtrate was centrifuged at 1,000*g* for 2 min to reduce disturbing chloroplast material (5). Cysteine in the preparation medium interfered with the measurements of Cyt *c* oxidase and NADPH:Cyt *c* oxidoreductase. The filtrate was therefore centrifuged at 200,000*g* for 60 min and activities in the resulting pellet were determined. IDH activity is expressed as nmol NADH produced min^{-1} mg^{-1} protein; Cyt *c* oxidase activity as nmol Cyt *c* oxidized min^{-1} mg^{-1} protein; glycolate oxidase activity as nmol O_2 consumed min^{-1} mg^{-1} protein; and NADPH:Cyt *c* oxidoreductase as nmol Cyt *c* reduced min^{-1} mg^{-1} protein. The recovery of Chl and protein is measured in preparations from 150 g leaves.

	Centrifugation	Phase Partition	Percoll Gradient
IDH			
% Filtrate	44 (35–56)	14 (11–17)	9 (7–13)
Specific activity		246 (195–297)	274 (248–295)
Cyt *c* oxidase			
% Filtrate	43 (30–54)	13 (10–15)	8 (6–11)
Specific activity		598 (410–783)	576 (371–765)
Glycolate oxidase			
% Filtrate	6.9 (4.9–8.3)	2.2 (1.6–2.7)	1.0 (0.6–1.3)
Specific activity		316 (217–538)	167 (101–237)
NADPH:Cyt *c*			
% Filtrate	12 (8–15)	1.4 (0.8–2.6)	0.4 (0.2–0.7)
Specific activity		3.1 (2.1–3.3)	1.3 (1.1–1.5)
Chl			
% Filtrate	2.5 (1.9–3.0)	0.06 (0.04–0.09)	<0.002
mg	2.9 (2.0–3.3)	0.09 (0.05–0.12)	<0.002[a]
Protein			
mg		7.9 (5.2–10.1)	4.9 (4.1–6.1)

[a] Approximate limit of detection.

Table III. *Enrichment of Mitochondria*

The enrichment of mitochondria relative to thylakoid membranes, peroxisomes and microsomes is expressed as ratios of recoveries of activities after the different steps of purification. The values are average ratios from at least five preparations. Figures in parentheses show the maximum and minimum values obtained from different preparations.

	Centrifugation	Phase Partition	Percoll Gradient
$\frac{\%\ IDH}{\%\ Chl}$	22 (17–27)	290 (220–398)	>4500[a]
$\frac{\%\ IDH}{\%\ Glycolate\ oxidase}$	7.2 (6.1–9.0)	6.7 (5.5–9.5)	12 (9–16)
$\frac{\%\ IDH}{\%\ NADPH:Cyt\ c}$	4.8 (3.0–7.6)	16 (8–24)	33 (18–59)
$\frac{mg\ protein}{mg\ Chl}$		102 (71–139)	>2450[a]

[a] Based on the approximate limit of detection of Chl.

gation. The intactness obtained in this study is considerably higher than reported earlier for mitochondria prepared from spinach (6, 7).

Table I shows the respiratory properties of the mitochondria after the phase partition and Percoll gradient centrifugation steps. The respiratory rates for the different substrates were increased 30 to 60% after Percoll gradient centrifugation; the specific activities for the mitochondrial enzymes tested were very little affected (Table II). This difference can be explained by effects of membrane integrity on respiratory activities. The highest increase in respiratory rate was obtained with glycine as substrate. This is in agreement with the findings that glycine oxidation in plant mitochondria shows an absolute demand on inner membrane integrity (17). After Percoll gradient centrifugation the RC and ADP/O ratios are somewhat increased as would be expected from the increased membrane integrity. The respiratory rates with the different substrates were always in the order NADH > succinate > glycine in our preparations. Jackson *et al.* (6, 7) reported the relative rates: NADH > glycine > succinate. In our opinion these differences probably reflect differences in the plant material. In comparison with the preparation by Jackson *et al.* (6, 7) the state three respiratory rates with NADH and succinate as substrate are higher in our preparation whereas the same rate was obtained with glycine as substrate. In Table II the recovery of mitochondrial and contaminating activities in the different steps of the preparation is summarized. The recovery of intact mitochondria is about 10% of the total amount of mitochondria. Realizing that a considerable fraction of the mitochondria is damaged during homogenization the true recovery of intact mitochondria is higher, and the overall yield seems good despite the fact that the preparation is a multistep procedure. The specific activity of IDH is slightly increased in the Percoll gradient centrifugation step, whereas the specific activity of Cyt *c* oxidase is slightly decreased. This indicates removal of mitochondrial membrane fragments in the Percoll gradient centrifugation.

The three purification steps complement each other as the separation in each step is based on different properties of the material. Centrifugation separates material mainly according to size, phase partition according to surface properties, and Percoll gradient centrifugation according to density differences. In order to remove completely the thylakoid contamination, all three purification steps must be used as differential centrifugation—phase partition (5) and differential centrifugation—Percoll gradient centrifugation (6) procedures both leave some Chl in the mitochondrial preparations. The peroxisomal contamination is mainly reduced in the centrifugation step (Table III). The enrichment is probably due to more extensive damage of peroxisomes than mitochondria during homogenization as little further enrichment is obtained by repeated centrifugations (7). The amount of microsomes is reduced in all three preparation steps, but most effectively by differential centrifugation and phase partition. For most studies the microsomal contamination in the final mitochondrial preparation should be of little significance.

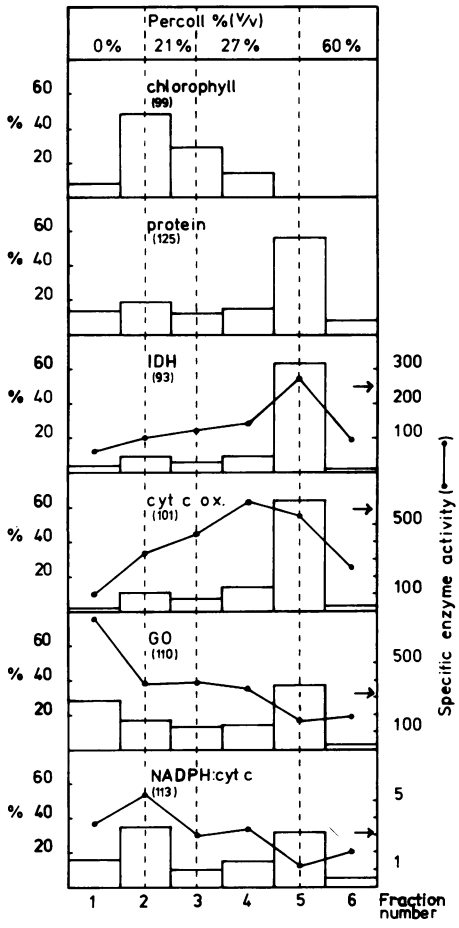

FIG. 2. Distribution of markers on a discontinuous Percoll density gradient. The recovery (histogram) and the specific activity (•—•) are mean values based on a minimum of four preparations. The amount of Percoll in the different layers of the gradient is shown (top). Dashed lines indicate interfaces. Fractions were withdrawn from the gradient as described. The composition of the gradient and the location of the fractions are also indicated in Figure 1. The values in parentheses under each marker show the total recovery in the gradient in percent. The specific activity before Percoll gradient centrifugation is indicated for each marker enzyme by an arrow. IDH activity is expressed as nmol NADH produced min^{-1} mg^{-1} protein and glycolate oxidase activity as nmol O_2 consumed min^{-1} mg^{-1} protein. Cyt *c* oxidase activity is expressed as nmol Cyt *c* oxidized min^{-1} mg^{-1} protein and NADPH: Cyt *c* oxidoreductase activity as nmol Cyt *c* reduced min^{-1} mg^{-1} protein.

Most mitochondrial preparations from green leaves are contaminated by peroxisomes. The use of substituted PEG in phase partition is potentially a very effective method for separating membraneous material and has, for example, been used for specific extraction of chloroplasts (8). Preliminary results in our laboratory show that with the use of this technique it may be possible to reduce considerably the peroxisomal contamination of the mitochondrial preparation.

The protein distribution profile after the Percoll gradient centrifugation is very similar to the distribution of mitochondrial enzyme activities (Fig. 2), indicating that the mitochondrial preparation after phase partition is fairly pure. The intactness after phase partition is also high, and the respiratory properties (Table I) are rather good. The preparation obtained by differential centrifugation and phase partition is thus sufficiently pure and intact for many functional studies. Taken together the three purification steps give a highly purified preparation of intact mitochondria with peroxisomes as the most significant contamination. The preparation should be very useful for studies of molecular composition and enzyme distribution.

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