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A procedure for the rapid preparation of mitochondria from rat liver

Peter H. REINHART, Wayne M. TAYLOR and Fyfe L. BYGRAVE
Department of Biochemistry, Faculty of Science, Australian National University, Canberra, A.C.T. 2600, Australia

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A technique for the rapid preparation of mitochondria from rat liver is described. Tissue fractionation is performed by a single centrifugation step with a discontinuous Percoll density gradient. Total preparation times of 5–6 min are achieved by using this method. The mitochondrial fraction obtained is relatively free of contaminating organelles, as judged by marker-enzyme activity determinations. Mitochondria isolated by Percoll-density-gradient centrifugation differ from mitochondria obtained by differential centrifugation [Taylor, Prpić, Exton & Bygrave (1980) *Biochem. J.* **188**, 443–450] in that the former exhibit a higher acceptor control ratio and a higher calcium content. Values obtained for the protonmotive force are not significantly different between the two preparations. The technique described may be widely applicable for studies requiring the rapid preparation of functionally intact and relatively uncontaminated mitochondria.

A number of differential centrifugation procedures have been developed for the preparation of rat liver mitochondria (Schneider & Hogeboom, 1950; Appelmans *et al.*, 1955; Johnson & Lardy, 1967; Schnaitman & Greenawalt, 1968; Harada & Sato, 1974; Bustamante *et al.*, 1977). Although these allow for the isolation of coupled mitochondria in high yields, the final fractions are usually contaminated to various degrees with other organelles, and require preparation times of between 1 and 5 h (Baudhuin & Beaufay, 1963; Harada & Sato, 1974).

Because these long preparation times may allow a significant redistribution of metabolites or ions between organelles, a number of techniques for the rapid fractionation of cells have been developed. These involve the disruption of cells by shear forces created by passing isolated liver cells through a small-diameter needle under high pressure (Tischler *et al.*, 1977), or by using digitonin (Zuurendonk & Tager, 1974; Booth & Clark, 1979), or by a combination of both procedures (Scott *et al.*, 1980; Murphy *et al.*, 1980). The subsequent fractionation is achieved by centrifuging through silicone oil at 12000 *g*. These new techniques, however, are not applicable to the fractionation of intact tissue, and as presently employed result in a crude 'heavy particulate' fraction, rather than a purified mitochondrial fraction (Murphy *et al.*, 1980; Scott *et al.*, 1980).

In the present paper we describe a rapid simple method for the isolation of relatively uncontaminated intact mitochondria from perfused rat liver by the use of a discontinuous density gradient of iso-osmotic Percoll. Mitochondrial integrity and purity were examined by determining the protonmotive force, the acceptor control ratio and the distribution of marker enzymes.

Experimental

Liver perfusion and homogenization

Livers of male Wistar-strain albino rats weighing 200–250 g were perfused with Krebs & Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) and containing 1.30 mM added $CaCl_2$, essentially as described by Reinhart *et al.* (1982). After a 15 min pre-perfusion period, the median lobe was rapidly excised, placed into a chilled glass/Teflon tissue disintegrator (size C; A. H. Thomas Co., Philadelphia, PA, U.S.A.) containing 10 ml of ice-cold homogenization medium {210 mM-mannitol, 60 mM-sucrose, 10 mM-KCl, 10 mM-sodium succinate, 1 mM-ADP, 0.25 mM-dithiothreitol and 0.1 mM-EGTA in 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid]/KOH buffer, pH 7.4} and homogenized by using two rapid strokes of a pestle driven at 900 rev./min. Succinate and ADP were present to maintain the protonmotive force during isolation of the mitochondria. In addition ADP has been shown to lower the rate of Ca^{2+} efflux in isolated mitochondria (Harris, 1979). For experiments in which the mitochondrial calcium content was determined, EGTA was replaced with 0.5 μM-

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Ruthenium Red and 1 mM-Nupercaine, to minimize mitochondrial Ca^{2+} uptake (Moore, 1971) and efflux (Dawson & Fulton, 1980) respectively during cell fractionation. The time taken to complete the tissue disruption was 4–6 s.

Isolation procedure

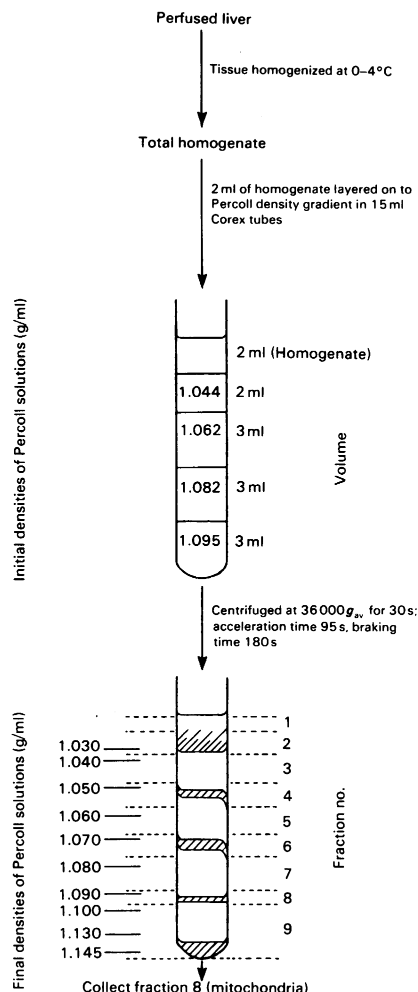
Commercially obtained Percoll was as a routine passed through a Chelex-100 column to remove contaminating cations. Step gradients, prepared in 15 ml Corex tubes, corresponded to approx. 52%, 42%, 31% and 19% (v/v) Percoll in medium containing 210 mM-mannitol, 60 mM-sucrose, 10 mM-KCl, 10 mM-sodium succinate, 1 mM-ADP and 0.25 mM-dithiothreitol in 10 mM-Hepes/KOH buffer, pH 7.4, to yield the densities shown in Scheme 1. Gradients were prepared on ice and used within 15 min of preparation. Density-marker beads were used to calibrate the gradients. The density values supplied for Percoll mixed with 0.25 M-sucrose were corrected for the slightly higher osmolarities of the solutions used in these experiments.

Portions of the liver homogenates (2 ml containing 127 ± 16 mg of protein; $n = 9$) were rapidly layered on to the gradients and immediately centrifuged at 20000 rev./min (36900 $g_{av.}$) for 30 s in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor. Preliminary experiments had established that this procedure is suitable for collecting mitochondria at the 42%/52% interface. Gradients were fractionated into nine fractions as shown in Scheme 1, and the distribution of organelles was analysed, with cytochrome *c* oxidase, glucose 6-phosphatase, 5′-nucleotidase, acid phosphatase and catalase as marker enzymes for mitochondria, endoplasmic reticulum, plasma membrane, lysozomes and peroxisomes respectively. For experiments in which only mitochondria were required, the gradient was aspirated to the 42%/52% interface, and the mitochondrial fraction was removed by automatic pipette and, without addition of medium, resuspended to a final protein concentration of 22.8 ± 3.1 mg/ml ($n = 12$). All operations were performed at 0–4 °C.

Mitochondria prepared by differential centrifugation (Taylor *et al.*, 1980) were isolated in homogenization medium, except for experiments involving the assay of total calcium, in which case EGTA was replaced with 1 mM-Nupercaine and 0.5 μM-Ruthenium Red.

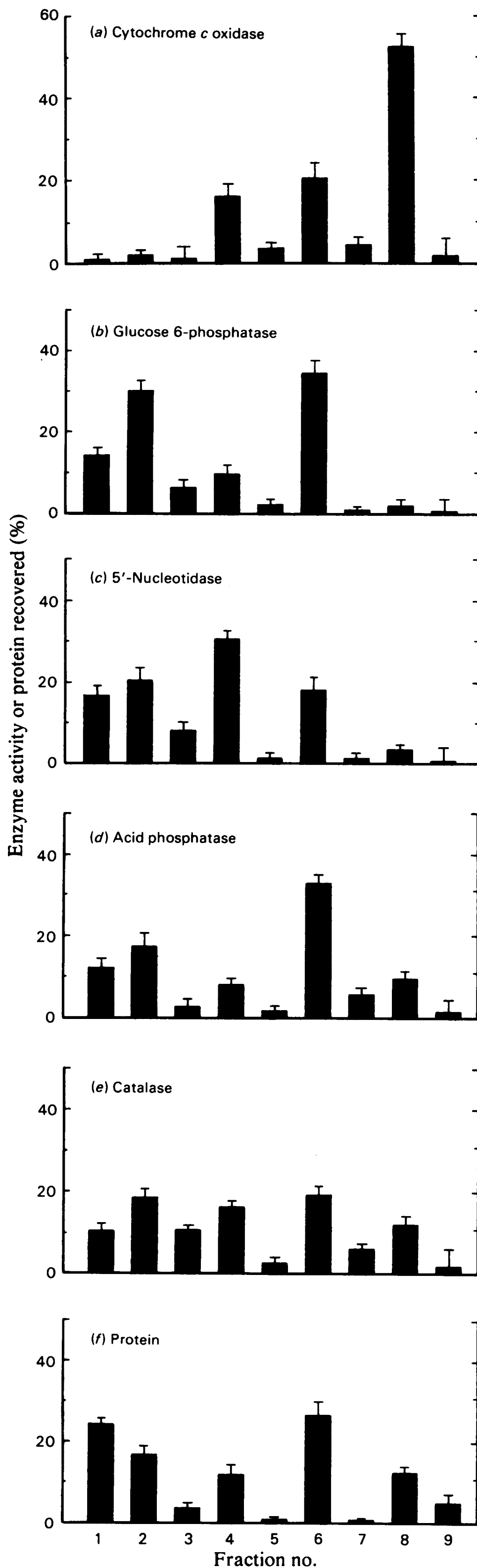
Analytical procedures

Respiration in the absence or in the presence of ADP was measured polarographically by using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambs., U.K.), in a medium containing 100 mM-sucrose, 50 mM-KCl, 10 mM-KH_2PO_4, 2 mM-$MgSO_4$, 1 mM-EDTA and 10 mM-sodium succinate in 15 mM-Tris/HCl buffer pH 7.4. The temperature was 25 °C. The components of the protonmotive force were determined by the ion-distribution technique of Nicholls (1974) as described by Taylor *et al.* (1980).



Scheme 1. *Flow diagram for the rapid preparation of mitochondria from perfused rat liver*
For full details see the Experimental section. As Percoll spontaneously forms a gradient when centrifuged at high speed, the final densities differ slightly from the starting densities.

Calcium concentrations were determined by atomic-absorption spectroscopy with a nitrous oxide/acetylene flame. Medical-grade gases only were used for these determinations. Glucose 6-phosphatase and 5′-nucleotidase activities were assayed as described by Bygrave & Tranter (1978), and cytochrome oxidase was assayed as described



by Bygrave *et al.* (1978). Acid phosphatase activity was measured by the method of Baudhuin (1974), after solubilization of approx. 100 μg of protein with 100 μl of 2% (w/v) Triton X-100 for 2 min. Inorganic phosphate was assayed by a modification (Dulley, 1975) of the procedure described by Baginski *et al.* (1967), after centrifuging the acid-treated samples at 12000 **g** for 2 min to remove the Percoll. Catalase activity was assayed by measuring the decrease in absorbance at 240 nm as described by Aebi (1974). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction 5; Sigma) as the standard. Results are expressed as the means ± S.E.M. for the numbers of independent experiments shown in parentheses.

Materials

Percoll and the density-marker beads were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ruthenium Red was from Sigma Chemical Co., St. Louis, MO, U.S.A. Chelex-100 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Corex centrifuge tubes were supplied by Du Pont Instruments, Newtown, CN, U.S.A. Nupercaine (dibucaine hydrochloride) was obtained from Astra Chemicals, Sydney, N.S.W., Australia.

Results and discussion

Scheme 1 is a flow diagram for the rapid preparation of mitochondria isolated from perfused liver. Purification is achieved by density-gradient centrifugation through iso-osmotic Percoll. This isolation procedure has several advantages. First, the preparation is rapid and involves a single centrifugation step. Fractionation is begun within 20 s of

Fig. 1. *Distribution of marker enzymes and protein in fractions obtained from Percoll density gradients*
Portions of liver homogenate (2 ml) were fractionated and marker-enzyme activities were assayed as described in the Experimental section. Results are expressed as percentages of the total activity recovered. Enzyme activities in the liver homogenate were 82.5 ± 9.6 ($n = 5$) nmol/min per mg of protein for cytochrome *c* oxidase, 34.6 ± 4.5 ($n = 5$) nmol of P_i/min per mg of protein for glucose 6-phosphatase, 41.6 ± 5.6 ($n = 4$) nmol of P_i/min per mg of protein for 5'-nucleotidase, 77.3 ± 9.4 ($n = 5$) nmol of P_i/min per mg of protein for acid phosphatase and 1.61 ± 0.32 ($n = 4$) ΔA_{240}^{1cm}/min per mg of protein for catalase. The recovery of marker enzyme activities, or of protein, in the nine fractions was at least 90%. Results are presented as means ± S.E.M. for between four and eight independent experiments.

Table 1. *Comparison of properties of different mitochondrial preparations*
Mitochondria were prepared either by Percoll-density-gradient centrifugation (Scheme 1) or by differential centrifugation (Taylor *et al.*, 1980). Respiration in the presence (State 3) or in the absence (State 4) of added ADP was measured polarographically. The acceptor control ratio is defined as the State 3/State 4 (Chance & Williams, 1956) respiration ratio. Determination of total calcium content and the components of the protonmotive force were performed as described in the Experimental section.

	Mitochondria prepared by	
	Density-gradient (Percoll) centrifugation	Differential centrifugation
State 3 respiration (ng-atoms of O/min per mg of protein)	108.11 ± 3.49 ($n = 6$)	86.56 ± 0.82 ($n = 15$)
State 4 respiration (ng-atoms of O/min per mg of protein)	17.64 ± 0.55 ($n = 6$)	20.56 ± 0.82 ($n = 15$)
Acceptor control ratio	6.15 ± 0.34 ($n = 6$)	4.20 ± 0.16 ($n = 15$)
ΔpH (mV)	76.7 ± 2.4 ($n = 4$)	76.2 ± 2.1 ($n = 6$)
ΔE (mV)	125.5 ± 2.0 ($n = 4$)	133.5 ± 3.3 ($n = 6$)
Protonmotive force (mV)	200.2 ± 4.1 ($n = 4$)	209.7 ± 4.1 ($n = 6$)
Calcium content (nmol/mg of protein)	6.75 ± 0.33 ($n = 6$)	4.95 ± 0.48 ($n = 5$)

excising the liver lobe and is complete within 6 min. Secondly, a mitochondrial fraction only minimally contaminated with other organelles is obtained (see below). Thirdly, mitochondria are not subjected to high hydrostatic pressures or hyperosmotic conditions, as may occur in sucrose-density-gradient centrifugation (Wattiaux *et al.*, 1971).

As shown in Scheme 1 and Fig. 1, the bulk of the mitochondria are recovered at a density of 1.085–1.095 g/ml (fraction 8). This density range is similar to those quoted for fat-cell mitochondria (Belsham *et al.*, 1980) and liver mitochondria (Jenkins *et al.*, 1979; Blume, 1979). The density steps were chosen in order to obtain a relatively pure mitochondrial fraction in high yield (30 mg of mitochondrial protein/g wet wt. of liver). This compares favourably with mitochondrial yields obtained by using differential centrifugation (Bustamante *et al.*, 1977). However, lower yields were obtained when, in an attempt to decrease contamination, the density of the 1.082 g/ml step was increased to 1.087 g/ml (results not shown).

Fig. 1 shows that fraction 8 is relatively free of contaminating endoplasmic reticulum or plasma membranes, as evidenced by the low recovery of glucose 6-phosphatase or 5′-nucleotidase activity. The distributions of acid phosphatase and catalase are far more heterogeneous for the density range 1.040–1.108 g/ml, and hence lysosomes and peroxisomes are a more significant contaminant of fraction 8. This extent of contamination by organelles of non-mitochondrial origin is comparable with, and in some cases less than, that which occurs in conventional mitochondrial preparations (see, e.g., Baudhuin & Beaufay, 1963; Harada & Sato, 1974; Gellerfors & Nelson, 1979; F. L. Bygrave & T. P. Heaney, unpublished work).

The data in Table 1 show that rapidly prepared mitochondria have a significantly higher acceptor control ratio than do mitochondria prepared by differential centrifugation. This was due largely to a higher rate of respiration in the presence of added ADP by rapidly prepared mitochondria, and is indicative of a higher state of coupling between oxidation and phosphorylation. The components of the protonmotive force (ΔE and ΔpH) were measured in the absence of any added substrate, and were not significantly different ($P < 0.1$) between the two preparations of mitochondria.

The calcium content of rapidly prepared mitochondria is significantly higher than that measured in mitochondria isolated by conventional means, emphasizing the possibility that the redistribution of ions, including Ca^{2+}, may occur during the isolation of mitochondria by conventional methods (even in the presence of 0.5 μM-Ruthenium Red and 1 mM-Nupercaine). Hence the rapid isolation procedure described should be useful for studying relatively rapid changes in the concentrations of mitochondrial metabolites and ions.

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