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A High-Yield Preparative Method for Isolation of Rat Liver Mitochondria

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A differential centrifugation method for the preparation of rat liver mitochondria is described, which results in final mitochondrial yields of at least 35 to 40 mg of mitochondrial protein per gram wet weight of liver. These mitochondria are shown to be functionally and ultrastructurally intact. They exhibit acceptor control ratios of 6 to 8 and ADP/O ratios near 2.0 with succinate as a substrate. In addition, they appear homogeneous by electron microscopy criteria. This method can be used to prepare rat liver mitochondria in high yield on either a large- or a small-scale basis.

Mitochondria isolated from rat liver offer many advantages over those from other tissues and organisms. The ready availability of the rat, the use of mild homogenization procedures in order to disrupt the relatively soft hepatic tissue, the homogeneity of the liver mitochondrial population, the availability of subfractionation procedures for rat liver mitochondria (1), the applicability of liver mitochondria as a normal control for those derived from hepatomas, and the central role played by liver in intermediary metabolism are some of the considerations that make rat liver a very suitable source for preparing mitochondria. The rat liver system has recently begun to be utilized for many molecular studies involving the mechanisms of ATP synthesis, ATP utilization, and the regulation of these processes. In recent years reliable procedures have been worked out for preparing from rat liver mitochondria the F_1-ATPase (2,3), an ATPase inhibitor peptide (4), and a partially purified preparation of the oligomycin-sensitive ATPase (5).

There are several differential centrifugation methods available for the isolation of rat liver mitochondria (1,6–10). All of these methods are quite reliable for obtaining mitochondria with good structural and functional integrity in the shortest possible time rather than achieving a maximal yield. However, when large-scale preparations are necessary as starting material for the purification of submitochondrial components, these methods are very expensive and time consuming since the mitochondrial yields are typically low. In this communication, we present a preparative-

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scale procedure for the isolation of rat liver mitochondria in yields significantly higher than the methods available to date. Furthermore, results are shown which indicate that the mitochondria obtained in high yield are functionally and structurally intact and have characteristics that are identical to mitochondria isolated by other methods.

MATERIALS

Male albino rats were purchased from the Charles River Co. (Wilmington, Massachusetts). The homogenization medium (H-medium) used throughout contains 220 mM mannitol, 70 mM sucrose, 2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 0.5 g/liter of defatted bovine serum albumin, pH 7.4 (1).

Sucrose (RNase free) was purchased from Schwarz/Mann. Mannitol and 2,4-dinitrophenol were obtained from Sigma Chemical Co. HEPES was purchased from Calbiochem. Adenosine diphosphate (ADP) was purchased from P–L Biochemicals, and defatted bovine serum albumin (BSA) from ICN Pharmaceuticals. All other reagents used were analytical grade or of the highest purity commercially available.

METHODS

Rats were sacrificed by a blow on the head, decapitated, and immediately exsanguinated. The livers were quickly removed, cleansed of connective tissue, fat, and blood, and minced with scissors to fragments smaller than 0.5 cm. A 25% (w/v) suspension of liver fragments was made in ice-cold H-medium and homogenized in a glass Thomas homogenizer by applying two strokes with a Teflon pestle rotating at about 1500 rpm. The cell fractionation scheme used is depicted in Fig. 1 where centrifugal forces are expressed in terms of time integrals of the sedimentation forces (11). All steps were carried out at 0–4°C. The homogenate was centrifuged at 1100*g* for 3 min (3300*g*-min). The resulting sediments were suspended in H-medium, homogenized by applying one stroke of the pestle rotating at 1500 rpm, and centrifuged at 1100*g* for 3 min. Two more successive washes of the sediments were performed in which the sediments were again resuspended, homogenized by applying one stroke of the pestle rotating at 1500 rpm, and centrifuged at 1100*g* for 3 min. The collected supernatants constitute the cytoplasmic fraction. This fraction was made up to a final volume of 10 ml per gram of original weight of liver and centrifuged at 6800*g* for 15 min (100,000*g*-min). The sediments were gently suspended by manual homogenization, pooled into half the original number of tubes, and centrifuged at 20,000*g* for 10 min (200,000*g*-min). The sediments were then resuspended into one-fourth the original number of tubes, and centrifuged at 3000*g* for 3 min. The resulting

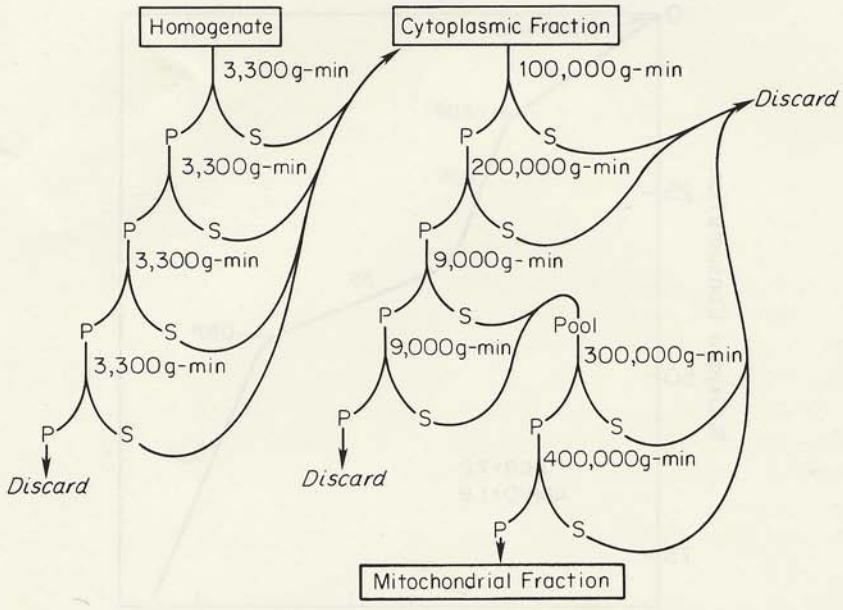

FIG. 1. Cell fractionation scheme. Suspensions of sediments resulting from the homogenate were performed by applying one stroke with a Teflon pestle rotating at 1500 rpm. Sediments resulting from the cytoplasmic fraction were suspended by manual homogenization. Abbreviations are: P = sediment, S = supernatant. Experimental details are described under Methods.

low-speed supernatants were saved, and the low-speed sediments were resuspended into one-eighth the original number of tubes and centrifuged at 3000*g* for 3 min. The low-speed sediments, containing any residual contamination carried from the nuclear fraction, were discarded and the pooled supernatants were centrifuged at 20,000*g* for 15 min (300,000*g*-min). At this point the resulting sediments were quite compact, and it was possible to begin separation of an upper loosely packed ("fluffy") layer by careful rinsing with H-medium. The rinsed sediments were then gently resuspended, pooled into one-eighth the original number of tubes, and centrifuged at 20,000*g* for 20 min (400,000*g*-min). The resulting sediments constituted the mitochondrial fraction and they were then further rinsed with H-medium, suspended, pooled, and taken to the desired final protein concentration.

Respiration rates and acceptor control ratios were determined polarographically with a Clark oxygen electrode, essentially as described by Estrabrook (12), in a 2.88-ml system containing 220 mM mannitol, 68 mM sucrose, 1.9 mM HEPES buffer, 2.4 mM potassium phosphate buffer, 0.5 mM sodium EDTA, 1.8 mM $MgCl_2$, 5.2 mM sodium succinate, and 0.7

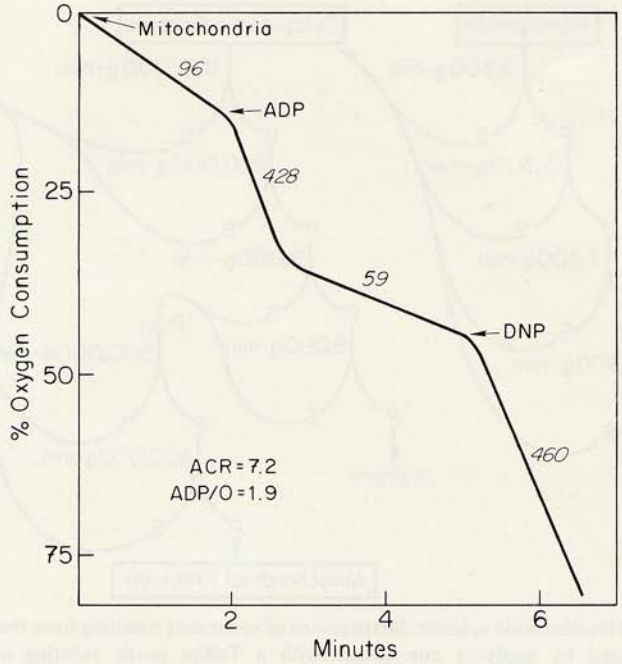

FIG. 2. Typical respiratory activity of rat liver mitochondria isolated by differential centrifugation. Respiratory substrate was 5.2 mM succinate. Respiratory rates are expressed in nanoatoms of O_2/min. Indicated additions are: 2.5 mg of protein, 450 nmol of ADP, and 100 nmol of DNP. Total oxygen dissolved in 2.87 ml of reaction volume is 1380 natoms of O_2. ACR = acceptor control ratio. Experimental details are described under Methods.

g/liter of defatted BSA, at pH 7.4 and 25°C. Mitochondria, ADP, and 2,4-dinitrophenol (DNP) were added at the concentrations noted in Fig. 2.

Protein concentration was determined by the biuret method (13) in a total volume of 3 ml containing 6.2 mM cholic acid, 1.67 N NaOH, and 4 mM $CuSO_4$. Absorbance was read at 540 nm after a 5-minute incubation period at 25°C. Biuret protein samples were standardized against 10 g/liter of BSA solutions.

Packed mitochondrial fractions were prepared for electron microscopy by fixation in 3% (w/v) glutaraldehyde for 2 hr at 4°C, followed by 1% (w/v) OsO_4 for 1 hr at 20°C. Fixed samples were sequentially dehydrated with ethanol and were embedded in a resin based in Epon 812. Thin sections were obtained with an LKB ultramicrotome, stained with 2% (w/v) uranyl acetate and lead citrate (14), and observed at 60 kV in a Jeol 100B electron microscope.

RESULTS

Figure 1 depicts the differential centrifugation scheme used in this study. The major features of this method are as follows: (a) several washing steps at low centrifugal forces are used for the separation of the nuclear fraction. This ensures an increased recovery of mitochondria that would have otherwise sedimented in the nuclear fraction. (b) The mitochondria are first sedimented from the cytoplasmic fraction by applying a low centrifugal force (100,000*g*-min). This is enough for sedimenting most of the mitochondria and at the same time ensures low contamination of the sediment with lysosomes and peroxisomes. (c) The three further washes of the mitochondrial sediment are performed with increasing centrifugal forces (Fig. 1) in order to form a well-packed mitochondrial sediment. (d) After the 300,000*g*-min centrifugation step, the mitochondrial sediment is quite compact, and an upper loosely packed (fluffy) layer can be readily separated from the compact sediment by careful rinsing with H-medium. The fluffy layer is thought to consist of some small mitochondria and the usual contaminants of mitochondrial fractions, namely lysosomes and peroxisomes.

Figure 2 shows the respiratory activity of the mitochondrial fraction. As can be seen, ADP markedly stimulates the normally slow rate of respiration for about 0.5 min. At this point, respiration returns to an even slower basal rate. The acceptor control ratio is 7.2 in this particular experiment and, in most other determinations, is within a range of 6.0 to 8.0 with succinate as a substrate. The calculated ADP/O ratio is 1.9 for the experiment shown in Fig. 2, a value that is very close to the theoretical ATP/O ratio of 2.0 with succinate as a substrate. Figure 2 also shows that the uncoupler 2,4-DNP can stimulate respiration to a rate slightly higher than that obtained through stimulation by ADP. These results strongly indicate that these mitochondria are functionally intact and that they have a well-coupled oxidative phosphorylation system.

Figure 3 is a representative electron micrograph of a mitochondrial fraction prepared by the "high-yield" differential centrifugation procedure. This subcellular fraction is shown to be highly enriched in mitochondria which have clearly distinguishable intact outer and inner membranes, defined cristae, an electron-dense matrix, and a visible intermembrane space. Few nonmitochondrial organelles can be detected in these electron micrographs.

Biochemical criteria also indicate that these mitochondrial fractions have very little contamination with other organelles. The relative specific activity (i.e., the ratio of specific activity of the fraction to specific activity of the homogenate) of acid phosphatase is one-seventh that of the

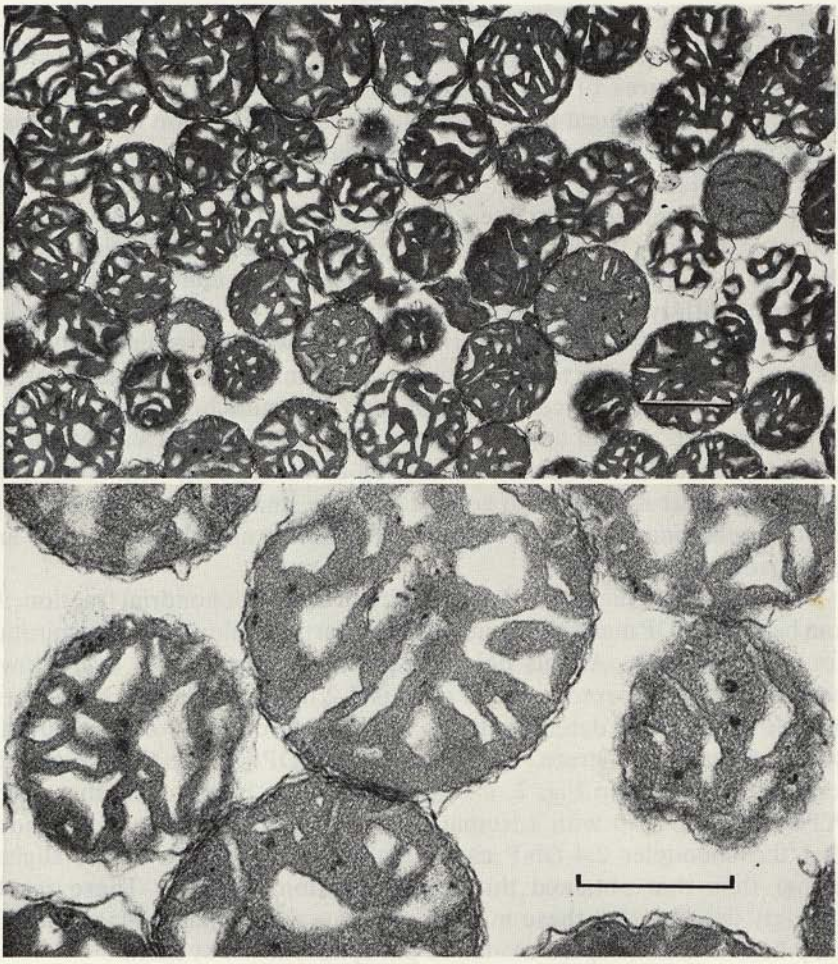

FIG. 3. Representative electron micrographs of the mitochondrial fraction. Mitochondria prepared by differential centrifugation were processed for electron microscopy as indicated under Methods and observed at original magnifications of under 10,000×. Upper panel, bar is 1 μm; lower panel, bar is 0.5 μm.

mitochondrial enzyme succinic dehydrogenase (data not shown); this implies that lysosomes are concentrated from the original homogenate only one-seventh as much as mitochondria. These results support the contention that the high-yield procedure provides mitochondria which are minimally contaminated with lysosomes, as are mitochondrial fractions prepared by most other methods (9). Should lysosome-free mitochondrial fractions be desired, the fraction described here can be treated with low digitonin concentrations as indicated by Lowenstein *et al.* (15).

DISCUSSION

A novel differential centrifugation method is described which provides functionally and ultrastructurally intact mitochondria from rat liver. The most significant feature of this method is that the final mitochondrial yield is significantly higher than that obtained by other methods (Table 1). There are several published methods currently being used for preparing mitochondria from rat liver (1,6–10). As shown in Table 1, the mitochondrial yields obtained by these methods are markedly lower than the yields obtained with the procedure presented in this communication. For instance, in our hands the classic method of Schneider and Hogeboom (6) yields about 20 mg of mitochondrial protein per gram wet weight of liver. The method described here, on the other hand, results in a yield of about 40 mg of mitochondria per gram wet weight of liver.

The net gain in mitochondrial yield provided by the procedure described here results from recovering mitochondria which sedimented in the nuclear fraction. This is supported by the fact that repeated suspension and homogenization of the nuclear sediments are the major experimental steps which distinguish the method described here from the procedures previously described for preparing rat liver mitochondria by differential centrifugation (1,6–10). The reason why such a significant proportion of the liver mitochondrial population sediments with the nuclear fraction remains unknown. The possibility that such mitochondria are larger than normal is not supported by our electron microscopic data (Fig. 3). Moreover, these mitochondria have an isopycnic density (data not shown) which is identical to that typically described for rat liver mitochondria (16). Rather, it would seem that some unbroken or partially fragmented cells may sediment with the nuclear fraction or that some mitochondria may adhere to nuclei or aggregate with them. In such cases,

TABLE 1

PROTEIN YIELDS OF MITOCHONDRIAL FRACTIONS FROM RAT LIVER PREPARED BY DIFFERENTIAL CENTRIFUGATION

Method	Mitochondrial protein[a] (mg/g of wet liver)
Schnaitman and Greenawalt (1)[b]	15
Schneider and Hogeboom (6)	20
Kaschnitz *et al.* (7)	10
Johnson and Lardy (8)	25
Appelmans *et al.* (9)	25
This investigation	40

[a] Refers to biuret protein determinations.
[b] Number in parentheses is reference.

repeated suspension and homogenization of the nuclear sediments (as carried out here) would be predicted to release additional mitochondria and result in higher final yields.

Significantly, we have noticed in recent studies that mitochondrial yields can be improved even further by working with a low starting amount of liver tissue. Thus, if only about 15 g of wet liver is used, the final mitochondrial yield is in the order of 50 to 55 mg of mitochondrial protein per gram wet weight of liver, a value twofold higher than the highest yield reported for other procedures.

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