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Oxidative Phosphorylation in Rat Liver Mitochondria Isolated by Rate Zonal Centrifugation: Examination of Ficoll Gradients and Subpopulations of Mitochondria

Cecil Bruce Pickett, Joseph Cascarano, and Richard Johnson

Department of Biology,
University of California, Los Angeles
Los Angeles, California 90024

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Abstract

In order to measure the parameters of oxidative phosphorylation it is necessary to isolate physiologically intact mitochondria. The isolation of rat liver mitochondria by rate zonal centrifugation utilizing iso-osmotic Ficoll gradients resulted in the uncoupling of oxidative phosphorylation in these organelles. Analysis of the Ficoll solutions used to construct the gradients indicated that the Ca^{2+} content (200–400 nmole Ca^{2+}/mg protein) was sufficiently high to cause an uncoupling of oxidative phosphorylation. Treatment of the Ficoll solutions with Amberlite MB-3 resin reduced the Ca^{2+} content to levels below the limit of determination of the assay procedure. This resulted in the retention of respiratory control (1.42) in rate-zonally centrifuged mitochondria. The addition of bovine serum albumin (100 mg %) to the Ficoll gradients increased the respiratory control index to 2.10. The increase is due to an elevation in state 3 respiration rather than any change in state 4 respiration. The addition of 200 mg % bovine serum albumin to the Ficoll gradient did not further enhance the respiratory control index.

Examination of subpopulations of rat liver mitochondria revealed that they are heterogeneous with regard to states 3 and 4 respiration, respiratory control indices, and ADP : O ratios. In mitochondrial subpopulations respiratory control indices ranged from 1.00 to 4.13 and

ADP : O ratios ranged from 1.22 to 1.83. This investigation defined a procedure for the isolation of physiologically intact mitochondria from rat liver homogenates.

Introduction

The ability to isolate physiologically intact mitochondria which are representative of the population found in tissue homogenates is invaluable in light of recent studies indicating that these organelles are biochemically heterogeneous [1–4]. Mitochondria isolated from tissue homogenates by equilibrium density centrifugation are exposed to hyperosmotic sucrose gradients and high hydrostatic pressure which structurally damage these organelles [5–10]. Although the use of hyperosmotic sucrose gradients does not greatly alter mitochondrial enzymatic activities, it does appear that oxidative phosphorylation is severely impaired [11–12]. As a result, whenever measurements of respiration, ADP : O ratios, and respiratory control indices are to be made, most investigators utilize differential centrifugation to isolate mitochondria. Since this procedure does not damage mitochondria, analyses involving physiological function and degree of intactness can be performed. However, differential centrifugation does result in a selective loss of both large and small mitochondria and, therefore, of populations which are biochemically dissimilar. As a result, the measurement of parameters normally used as indices of mitochondrial function may not be an adequate reflection of values which would be obtained if the total mitochondrial population were sampled.

A technique which can be employed to isolate mitochondria from tissue homogenates without damage or severe loss of these organelles is rate zonal centrifugation utilizing iso-osmotic Ficoll gradients. Dimino and Hoch [12] have found that iso-osmotic Ficoll gradients as well as low rotor speeds (5000 revs/min) and short centrifugation times (5 min) were required in order to maintain respiratory control in rat liver mitochondria after rate zonal centrifugation. Since this study subjected mitochondria to zonal centrifugation after they had initially been isolated by differential centrifugation, the low rotor speed and short centrifugation time were suitable for adequate resolution of mitochondria from other cellular organelles. However, neither of these parameters is sufficient to allow for the resolution of mitochondria directly from tissue homogenates. Both the centrifugation time and rotor speed must be increased.

Although Ficoll (a copolymer of sucrose and epichlorohydrin with a molecular weight of 400,000) can be used to construct iso-osmotic gradients, investigators have reported severely diminished P : O ratios of mitochondria isolated in these gradients [13, 14]. It would appear that

Ficoll induces a major alteration in oxidative phosphorylation. Whether this alteration is induced by structural damage to the mitochondria during centrifugation or by some inherent property of the Ficoll is unknown.

The objective of this investigation was to develop a procedure of rate zonal centrifugation which would provide physiologically functional mitochondria isolated directly from whole tissue homogenates. First, experiments were conducted to examine oxidative phosphorylation in rat liver mitochondria isolated by rate zonal centrifugation utilizing gradients constructed from (a) sucrose, (b) Ficoll, (c) Ficoll treated with ion exchange resin (deionized Ficoll), and (d) deionized Ficoll plus bovine serum albumin. Second, a gradient constructed of deionized Ficoll plus bovine serum albumin was utilized to examine oxidiative phosphorylation in subpopulations of rat liver mitochondria.

Materials and Methods

Preparation and Rate Zonal Centrifugation of Liver Whole Homogenates

Male Sprague-Dawley rats, 280–290 g, were used in this study. Animals were decapitated and the liver immediately excised and placed in ice-cold 0.15 M NaCl. The liver was weighed, sliced, and a 15% (w/v) homogenate prepared in 0.25 M sucrose, 0.15 mM EDTA, and 0.01 M Tris-HCl (pH 7.4) using a Potter–Elvehjem glass homogenizer and a motor-driven Teflon pestle. The homogenate was filtered through four layers of medium cheesecloth and then through two layers of fine cheesecloth.

Fractionation of liver whole homogenate was done with the SZ-14 reorienting zonal rotor in a Sorvall RC2-B centrifuge [15]. A solution of 60% (w/v) sucrose was utilized for the cushion. Ficoll was dissolved in 8.5% (w/w) sucrose solutions and utilized to prepare a linear density gradient consisting of 1.5–11.5% (w/w) Ficoll (Pharmacia Fine Chemicals, Piscataway, New Jersey). The Ficoll solutions were treated with Amberlite MB-3 resin (25 g/600 ml of solution) for 2 hr, filtered, and Tris-HCl added to a final concentration of 0.01 M, pH 7.4. Bovine serum albumin, fraction V, was added to the Ficoll solutions to achieve a final concentration of 100 mg %.

The cushion (150 ml) followed by the gradient (1050 ml) was introduced into the rotor (rotating at 3000 revs/min) by means of a variable-speed Masterflex pump. Using a hypodermic syringe, a 25-ml sample of the whole homogenate followed by 20 ml of overlay (0.125 M sucrose, 0.01 M Tris-HCl buffer pH 7.4, 0.075 mM EDTA) was quickly sprayed through the opening of the rotor top onto the gradient and then centrifuged for 10 min at 10,000 revs/min (7600 × g_{av}). The rotor was decelerated, and after

it stopped the contents were collected in 40-ml fractions. Samples from the gradient were immediately taken for assays of marker enzymes and determinations of refractive index. Refractive indices were determined with an Abbe Refractometer.

Determination of the Distribution of Mitochondria in the Ficoll Gradient and the Degree of Contamination of the Mitochondrial Fractions with Other Cellular Organelles

The localization of mitochondria in the gradient was determined by the distribution of cytochrome oxidase. Cytochrome oxidase activity was assayed by the method of Wharton and Tzagloloff [16]. The reaction mixture contained 0.01 M potassium phosphate buffer (pH 7.0), 50 μM cytochrome *c*, and a sample of each fraction diluted in 0.01 M potassium phosphate buffer (pH 7.0). The rate of oxidation of reduced cytochrome *c* was followed at 550 nm in a Cary 15 split beam recording spectrophotometer.

Microsomal contamination of the mitochondrial fractions was determined by analyzing all fractions for glucose-6-phosphatase activity. Activity [17] was measured by determining the appearance of orthophosphate in the incubation medium. The incubation medium (final volume 2.0 ml) consisted of 75 mM sodium cacodylate buffer (pH 6.3), 10 mM glucose-6-phosphate, and a 1.0 ml sample of each fraction. The buffer and enzyme were preincubated at 30°C for 10 min. The reaction was initiated by the addition of substrate and the incubation continued for 10 min. Reaction was stopped by the addition of 1.0 ml of 15% (w/v) trichloroacetic acid cooled to 0–1°C, and the sample centrifuged at 4°C. P_i was determined in the supernatant by the method of Chen et al. [18]. Plasma membrane contamination of the mitochondrial fractions was determined by assaying all fractions for 5′-nucleotidase activity. Activity [19] was measured by determining the appearance of orthophosphate in the incubation medium. The incubation medium (final volume 2.0 ml) contained 100 mM KCl, 10 mM $MgCL_2$, 0.222 M Tris-HCl buffer (pH 7.4), 1.5 mM ATP, and 1.0 ml of each fraction from the gradient. The incubation procedure and the analysis of P_i in the supernatant were identical to that described for glucose-6-phosphatase. An orthophosphate blank was determined for each fraction at zero time and subtracted from the value obtained for each enzyme assay. Lysosomal contamination was determined by assaying each fraction for acid phosphatase activity by the method of Neil and Horner [20]. Activity was measured by the appearance of *p*-nitrophenol in the medium. The incubation medium (final volume 2.0 ml) consisted of 0.1 M sodium acetate buffer (pH 5.0) with 0.1% Triton X-100,

5 mM *p*-nitrophenyl phosphate, and a 1.0-ml sample of each fraction of the gradient. The enzyme, buffer, and Triton X-100 were preincubated at 30°C for 10 min. The substrate was added and the incubation continued for 10 min. The reaction was stopped by the addition of 10.0 ml of 0.04 N NaOH and the color read at 410 nm.

Determination of Oxidative Phosphorylation

For assessment of the experimental gradients, fractions #10–22 of the gradient were pooled and centrifuged at $7600 \times g_{av}$ for 15 min in a Sorvall SS-34 rotor to pellet the mitochondria. The pellets were resuspended in 0.25 M sucrose, 1 mM EDTA, and 0.01 M Tris-HCl buffer (pH 7.4). Mitochondrial respiration was measured polarographically at 30°C with an oxygen electrode apparatus manufactured by Rank Brothers, Cambridge, England. The incubation medium (1.6 ml) contained 0.3 M mannitol, 10 mM KCl, 0.25 mM EDTA, and 2.5 mM $MgCl_2$ in 10 mM potassium phosphate buffer (pH 7.4). To the incubation medium, mitochondrial suspension (2.0–4.0 mg protein), 100 µl of 20 mM succinate, and 100 µl of 0.20 mM ADP were successively added. States 3 and 4 respiration, ADP : O ratio, and respiratory control indices were measured by the method of Chance and Williams [21]. After selection of an optimal gradient, fractions #10–22 were individually centrifuged and mitochondria of each fraction separately analyzed as already indicated.

Determination of Ca^{2+}

Calcium was determined by a modification of the fluorometric titration method of Borle and Briggs [22]. A Calcein alkaline buffer was prepared by dissolving 200 mg of a 20 g KCl–200 mg Calcein (Sigma Chemical Company, St. Louis, Missouri) powdered mixture in 10 ml of alkaline buffer. The buffer was prepared by the addition of 200 ml of 0.1 M glycine to 800 ml of 0.1 M KOH. To 250 µl of the Calcein alkaline buffer was added to 2.9 ml of double-distilled water, and the blank fluorescence was measured. To this reaction mixture 100 µl of standard or unknown was added. The fluorescence of this solution was titrated to the blank value with 0.25 mM EDTA. The amount of EDTA required to titrate the sample is directly proportional to the Ca^{2+} content.

Results

The distribution of rat liver mitochondria in the Ficoll–sucrose gradient was determined by assaying for cytochrome oxidase. Mitochondria were

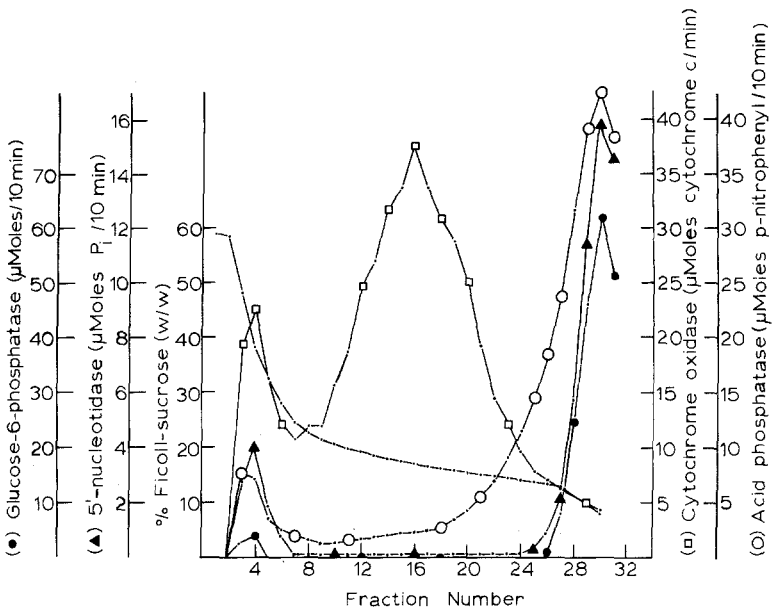

Figure 1. Distribution of marker enzymes in fractions from zonal centrifugation of a sample of liver whole homogenate utilizing iso-osmotic Ficoll–sucrose gradients. The centrifugation and assay procedures are described in the Materials and Methods section. The results presented are of a typical control experiment: · – ·, Ficoll–sucrose gradient; □, cytochrome oxidase; ○, acid phosphatase; ▲, 5'-nucleotidase; ●, glucose-6-phosphatase.

primarily localized in fractions #10–22 (Fig. 1). There was no apparent contamination of the mitochondrial fractions by either plasma membranes or microsomes as determined by 5'-nucleotidase and glucose-6-phosphatase, respectively (Fig. 1). Lysosomal contamination was minimal in the mitochondrial fractions. Less than 13% of the total lysosomal activity occurred in fractions #10–22.

When Ficoll was used to construct iso-osmotic gradients, the isolated mitochondria did not retain respiratory control (Table I). The loss of respiratory control was also found to occur during the isolation of mitochondria in a 14.5–45% (w/w) sucrose gradient (Table I).

Since some commercially obtained sucrose (used in the synthesis of Ficoll) can contain significant amounts of Ca^{2+} [23], we decided to assay for Ca^{2+} in the Ficoll solutions. We found that, depending on the lot of Ficoll used for making gradients, the 11.5% (w/w) Ficoll has a Ca^{2+} content of between 52 and 102 nmole Ca^{2+}/ml of solution and the 1.5% (w/w) Ficoll has a Ca^{2+} content of between 9 and 20 nmole Ca^{2+}/ml of solution (Table II). The 8.5% (w/w) surcrose in which the Ficoll is dissolved has no

TABLE I. The effect of gradients constructed from surcrose, Ficoll, deionized Ficoll, and deionized Ficoll plus bovine serum albumin on oxidative phosphorylation of isolated rat liver mitochondria [a]

Gradient solution	nmole O_2/min/mg protein			
	RCI	State 3	State 4	ADP : O
Sucrose	1.00	—	—	—
Ficoll	1.00	—	—	—
Ficoll (treated with Amberlite MB-3)	1.42	40.7	28.7	1.15
Ficoll (treated with Amberlite MB-3 + 100 mg % BSA	2.10	63.0	30.0	1.67
Ficoll (treated with Amberlite MB-3 + 200 mg % BSA)	2.14	68.5	32.0	1.61

[a] Livers from four animals were pooled for all determinations.

appreciable content of Ca^{2+} (Table II). In addition, no significant amounts of Ca^{2+} were found in the 14.5% or 45% (w/w) sucrose (Table II).

Upon treatment of the Ficoll solutions with Amberlite MB-3 resin, the Ca^{2+} content was reduced to levels which were not within the detectable limits of the assay procedure (<5 nmole/ml solution, Table II). When mitochondria were isolated in the treated Ficoll, respiratory control was maintained (Table I).

TABLE II. Ca^{2+} content of sucrose, Ficoll, and deionized Ficoll

Gradient solution		Treatment with Amberlite MB-3	
		Untreated [a]	Treated [a]
11.5% (w/w) Ficoll + 8.5% (w/w) sucrose	Lot 1	102	<5
	Lot 2	96	<5
	Lot 3	52	<5
1.5% (w/w) Ficoll + 8.5% (w/w) sucrose	Lot 1	20	<5
	Lot 2	15	<5
	Lot 3	9	<5
8.5% (w/w) sucrose		<5	
14.5% (w/w) sucrose		<5	
45.0% (w/w) sucrose		<5	

[a] nmole Ca^{2+}/ml solution.

The addition of bovine serum albumin (fraction V) to the gradient (final concentration of 100 mg %) resulted in an enhancement of the respiratory control index to 2.10 (Table I). Addition of bovine serum albumin to untreated Ficoll did not normally result in the maintenance of respiratory control. The enhanced respiratory control index is due to an elevation of state 3 (succinate as substrate) respiration (Table I) rather than any change in state 4 respiration. The ADP : O ratio was also markedly enhanced after the addition of bovine serum albumin (Table I). Increasing the bovine serum albumin concentration to 200 mg % did not result in a further enhancement of the respiratory control index, ADP : O ratio, or state 3 respiration over values obtained with 100 mg % bovine serum albumin (Table I).

After the isolation of liver mitochondria by zonal centrifugation, the respiratory control indices were lower than those obtained from nitochondria isolated by differential centrifugation. Therefore, we decided to determine if the isolation of mitochondria by zonal centrifugation resulted in a general lowering of respiratory control in the total mitochondrial population or whether subpopulations existed which were heterogeneous with regard to this parameter. For this reason, fractions #10–22 were individually centrifuged and the mitochondria of each fraction analyzed separately for respiratory activity (Table III). The respiratory control indices and ADP : O ratios were maximal in fraction 18

TABLE III. Oxidative phosphorylation in subpopulations of rat liver mitochondria fractionated by zonal centrifugation [a]

Fractions	nmole O_2/min/mg protein			
	RCI	State 3	State 4	ADP : O
10	1.00	—	—	—
11	1.00	—	—	—
12	1.22	34.6	25.8	1.22
13	2.48	65.9	25.7	1.54
14	2.63	50.1	18.8	1.61
15	2.73	57.8	21.8	1.68
16	3.23	66.5	20.5	1.75
17	3.78	63.7	16.9	1.79
18	4.13	70.3	17.1	1.83
19	3.51	66.2	18.9	1.75
20	3.04	66.9	22.1	1.68
21	2.51	66.6	26.7	1.63
22	2.27	56.9	25.3	1.63

[a] Livers from four animals were pooled for this determination. The experiment was repeated three times and the values given are those of a representative experiment.

(RCI = 4.13; ADP : O = 1.83) and were gradually diminished on either side of this fraction. State 3 respiration increased rapidly, reaching 94% of its maximum rate in fraction 13 followed by a sharp decline in fraction 14 (Table III). In fraction 16, state 3 respiration had returned to its maximum rate and remained at this rate through fraction 22. State 4 respiration was maximal in fractions 12–13 and 21–22, and was diminished in the intermediate fractions, being lowest in fractions 17 and 18 (63 and 64% of the maximum rate, respectively). These data show that mitochondria exhibit considerable heterogeneity and that when fractions were pooled (Table I) the low respiratory control indices were a reflection of the total mitochondrial population and not of a select group.

Discussion

Since functionally intact mitochondria cannot be isolated from tissue homogenates by zonal centrifugation utilizing linear sucrose gradients, we decided to construct an iso-osmotic gradient comprised of Ficoll to isolate rat liver mitochondria. Although Ficoll has little osmotic effect, we found that oxidative phosphorylation was severely impaired. It appears that the Ca^{2+} concentration is sufficiently high in commercially obtained Ficoll to induce the uncoupling of oxidative phosphorylation. After the treatment of Ficoll solutions with Amberlite MB-3 resin, the Ca^{2+} content was lowered to levels which could not be determined by the assay procedure (<5 nmole/ml of solution). Concomitant with the reduction of Ca^{2+} was the maintenance of respiratory control in isolated rat liver mitochondria. The addition of bovine serum albumin to the gradient resulted in an enhancement of state 3 respiration, respiratory control index, and ADP : O ratio.

Mitochondrial Ca^{2+} transport is an energy-requiring process which gives rise to a stimulation of oxygen consumption [24]. Dargel [25] has observed that succinate oxidation was uncoupled when rat liver mitochondria were preincubated in Ca^{2+} before the addition of succinate and rotenone. About 40 nmole Ca^{2+}/mg protein were required to cause the complete release from respiratory control. Since some sucrose can contain significant amounts of Ca^{2+} [23], Ficoll solutions were analyzed for Ca^{2+} content. In this investigation we found that the amount of Ca^{2+} (~200–400 nmole Ca^{2+}/mg protein) is sufficient to cause the release from respiratory control which we have observed. It is interesting to note that the sucrose solutions used to construct linear density gradients did not contain any significant amount of Ca^{2+}. Therefore, the possibility that Ca^{2+} induces the uncoupling of mitochondria isolated in our sucrose gradients is unlikely.

Since different lots of Ficoll can contain different amounts of Ca^{2+}, it is

likely that some lots may be sufficiently low in Ca^{2+} so as not to induce the uncoupling of oxidative phosphorylation. However, it generally appears that the Ca^{2+} content is high enough to warrant the use of some method to remove it. We have found that the most convenient method is to add Amberlite MB-3 resin to the Ficoll solutions. The resin reduced the Ca^{2+} content to levels which cannot be determined by the assay method. In addition, the resin can be easily removed by filtration. Concomitant with the reduction of Ca^{2+} is the maintenance of respiratory control in isolated mitochondria. The possibility that some other ionic contaminant of Ficoll induces the release of respiratory control cannot be eliminated, although it appears that Ca^{2+} is the most likely agent.

Although respiratory control could be maintained after reduction of Ca^{2+}, the mitochondria were only loosely coupled. We found that the addition of bovine serum albumin (fraction V) to the gradient resulted in an increased state 3 respiration rate which further enhanced the respiratory control index and ADP : O ratio. Since bovine serum albumin binds free fatty acids which uncouple oxidative phosphorylation [26], it seems reasonable to suggest that they may be partially responsible for the release from respiratory control. Whether the free fatty acids are liberated through the activation of a mitochondrial phospholipase during centrifugation is unknown.

Even though the respiratory control index was enhanced in mitochondria by pretreatment of the Ficoll solutions and the addition of bovine serum albumin, the values we obtained were still lower than those which could be obtained with mitochondria isolated by differential centrifugation. However, differential centrifugation results in a loss of mitochondria in the nuclear and postmitochondrial fractions [7]. Therefore, data for parameters of oxidative phosphorylation obtained from differential centrifugation would be an adequate representation of the total population only if the mitochondria were homogeneous. When we conducted respiratory analyses on individual fractions obtained by rate zonal centrifugation, we found that subpopulations of mitochondria are heterogeneous with regard to states 3 and 4 respiration (succinate as substrate), respiratory control indices, and ADP : O ratios. Mitochondria from the intermediate fractions (15–20) have respiratory control indices and ADP : O ratios that are very similar to determinations conducted on mitochondria isolated by differential centrifugation. Dimino and Hoch [12] have isolated rat liver mitochondria by differential centrifugation and obtained a respiratory control index of 2.75. They further reported a respiratory control index of 2.61 in mitochondria after rate zonal centrifugation in iso-osmotic Ficoll–sucrose gradients. Mitochondria in the latter experiment had initially been isolated by differential centrifuga-

tion before being subjected to zonal centrifugation. In view of the demonstrated heterogeneity, mitochondria collected by differential centrifugation are not representative of the total population but appear to be a segment of those collected by our preparation in fractions #15–20.

The heterogeneity in respiratory activity obtained in this study in subpopulations of mitochondria cannot be ascribed to artifact arising from preparative factors or conditions. For example, the nature of the heterogeneity obtained, high ADP : O ratios, or respiratory control indices in the intermediate fractions and diminished ratios in the bordering fractions, would rule out damage caused by such factors as Ficoll concentration or shear forces to which these subpopulations of mitochondria had been subjected during centrifugation. Support for the fact that mitochondria can be functionally different also comes from a number of studies demonstrating biochemical heterogeneity. Wilson and Cascarano [4] have shown that rat liver mitochondria isolated by rate zonal centrifugation in iso-osmotic Ficoll–sucrose were heterogeneous with regard to cytochrome *b* concentration, succinic dehydrogenase, and α-glycerophosphate dehydrogenase activities, but were similar with regard to the concentration of cytochrome $a + a_3$ and NADH dehydrogenase activity. They further demonstrated that the biochemical heterogeneity was not due to preparative damage or contamination. More recently, Thomson et al. [3] utilizing iso-osmotic D_2O gradients, and Weiss et al. [27] utilizing iso-osmotic Ficoll gradients, have also reported biochemical differences in subpopulations of rat liver mitochondria.

The ability to isolate functionally intact mitochondria by rate zonal centrifugation eliminates the need to use differential centrifugation to isolate these organelles whenever functional parameters are determined. Since our procedure does not result in a selective loss of large or small mitochondria, the population isolated is more representative of the population of mitochondria found in tissue homogenates. Moreover, the mitochondria are appreciably free from contamination by microsomes, lysosomes, and plasma membranes. As a result, the repeated washing and centrifugation required to purify mitochondria isolated by differential centrifugation can be avoided. More importantly, however, the mitochondria are sufficiently resolved by our procedure whereby subpopulations of mitochondria differing in sedimentation coefficients can be isolated and characterized to determine if any functional differences occur between them.

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