

## ISOLATION OF HIGHLY PURIFIED MITOCHONDRIA FROM RAT PANCREAS

ADRIANA HODĂRNĂU, SILVIA DANCEA, and OCTAVIAN BĂRZU. From the Department of Biochemistry and the Laboratory of Electron Microscopy, Medical and Pharmaceutical Institute, Cluj, Romania

### INTRODUCTION

The exocrine pancreas, an active secretory gland, is a suitable model for studies concerning the structure of cell organelles and the coupling of energy production with different biosynthetic processes (1, 2). However, the investigation of isolated pancreas mitochondria is not often performed because of the many difficulties in obtaining reproducible preparations with well-preserved structure and function (3, 4).

This work is an attempt to find optimal con-

ditions for the isolation of highly purified rat pancreas mitochondria, suitable for biochemical and electron microscope investigation. The small amount of mitochondria obtained is not a serious limitation, considering the recent advances in the study of the respiratory chain in minute amounts of biological materials (5-8).

### MATERIALS AND METHODS

Male and female albino rats weighing between 150 and 180 g were fasted for 18-20 h. The pancreas was removed under ether anesthesia, rinsed and

cooled in ice-cold homogenization medium, cut into small pieces, and homogenized with 0.5 ml of a medium containing 192 mM mannitol, 58 mM sucrose, 2 mM Tris-HCl, pH 7.4, 0.5% BSA<sup>1</sup>, and 0.5 mM EDTA. The homogenate was diluted to 15 ml with the same medium and centrifuged at 650 *g* for 7 min. The resulting supernate was centrifuged at 8,000 *g* for 10 min and the surface of the mitochondrial pellet was rinsed twice with 0.5 ml of medium. The pellet was resuspended in 5 ml of medium, slowly homogenized, and centrifuged again at 8,000 *g* for 10 min. The surface of the pellet was rinsed once with 0.5 ml of medium free of BSA and resuspended in a final volume of 0.2 ml (about 12 mg of protein/ml). RNA and protein content of mitochondria were determined by the procedures of Schneider (9) and Gornall et al. (10), respectively. Oxygen uptake of mitochondria was determined by the spectrophotometric method (5, 6), while ATP hydrolysis was estimated from released phosphate (11). Other mitochondrial enzymes were assayed by procedures described in the literature, as follows: malate dehydrogenase (MDH), isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), 12; cytochrome oxidase, 13;  $\beta$ -hydroxybutyrate dehydrogenase (HBDH), 14; fumarase, 15; and monoamine oxidase (MAO), 16 and 17. Samples were prepared for electron microscopy as follows: 5  $\mu$ l of mitochondrial suspension (about 0.05 mg of protein) were fixed for 1 h in 5% glutaraldehyde (0.2 ml of solution made in mannitol-sucrose-Tris medium) and centrifuged in microtubes (5  $\times$  20 mm) for 10 min at 3000 rpm. The pellet was washed twice with mannitol-sucrose-Tris medium, and postfixated for 1 hr in 1% OsO<sub>4</sub> in 0.1 M phosphate buffer, pH 7.0. After dehydration in an acetone series, the pellet was embedded in Vestopal W (Madame Martin Jaeger, Geneva). Thin sections were double stained with uranyl acetate and alkaline lead citrate and examined with an Hitachi HU-11 microscope.

## RESULTS AND DISCUSSION

Fig. 1 is an electron micrograph of isolated rat pancreas mitochondria at low magnification. A good preservation of mitochondrial integrity, the uniformity of their conformation state, and the absence of zymogen granules can be observed. At higher magnification, the presence of the outer membrane can be detected in most mitochondria (Fig. 2). The integrity of the outer membrane is

<sup>1</sup> Abbreviations used in this paper: BSA, bovine serum albumin; DNP, 2,4-dinitrophenol; HBDH,  $\beta$ -hydroxybutyrate dehydrogenase; ICDH, isocitrate dehydrogenase; MAO, monoamine oxidase; MDH, malate dehydrogenase; SDH, succinate dehydrogenase.

proved also by a very low rate of the oxidation of ascorbate with exogenous cytochrome *c* (18). As an indicator of microsomal contamination RNA content of organelles ( $18.6 \pm 2.2 \mu\text{g}/\text{mg}$  of protein) was significantly lower than that reported by Honjo et al. (3) for guinea pig pancreas mitochondria. The respiratory activity at 37°C of such a preparation in a standard assay medium (5) with succinate as substrate is 100 ng O atoms/min/mg of protein, P/O ratio being 1.8, and the respiratory acceptor index (the ratio between the respiratory activity after and before ADP addition) is higher than 6. Mg<sup>2+</sup>- and 2,4-dinitrophenol (DNP)-stimulated ATPases were used as very sensitive indicators of mitochondrial integrity (14). As shown in Table I, Mg<sup>2+</sup> stimulates the latent ATPase threefold, whereas stimulation by DNP is much higher. The addition of BSA to the incubation medium markedly decreases latent and Mg<sup>2+</sup>-activated ATPases, increasing at the same time the DNP-activated ATPase. This spectacular effect of BSA on both Mg<sup>2+</sup>- and DNP-stimulated ATPases, in agreement with the previous results obtained by Benga et al. (19) with isolated human liver mitochondria, appears to be due in part to the removal of free fatty acids. "Aged" mitochondria (6 h at 0°C) have almost completely lost the sensitivity of ATPase to DNP stimulation, but exhibit an important increase in Mg<sup>2+</sup>-activated ATPase. BSA added to the reaction medium partly restores the sensitivity to DNP, but was ineffective in restoring the initial values of Mg<sup>2+</sup>-activated ATPase. Mitochondria aged 6 h at 0°C in a medium containing 0.4 mM Nupercaine + 0.5% BSA have the same ATPase activity as the freshly isolated organelles. Nupercaine, a potent inhibitor of mitochondrial phospholipases, was shown to prevent the alteration of rat liver mitochondria during storage at 0°C (20). This observation provides a basis for obtaining a "confidence time interval" of at least several hours for the investigation of different parameters of pancreas mitochondrial functions.

The activities of the other mitochondrial enzymes investigated were closely similar to those of rat liver mitochondria, except for ICDH and HBDH, the latter being almost absent in pancreas mitochondria (Table II). Although the activity of ICDH is twofold higher in pancreas mitochondria, the respiratory activity with isocitrate is relatively weak. This may be due to the low ratio (0.08) of activities of the two kinds of

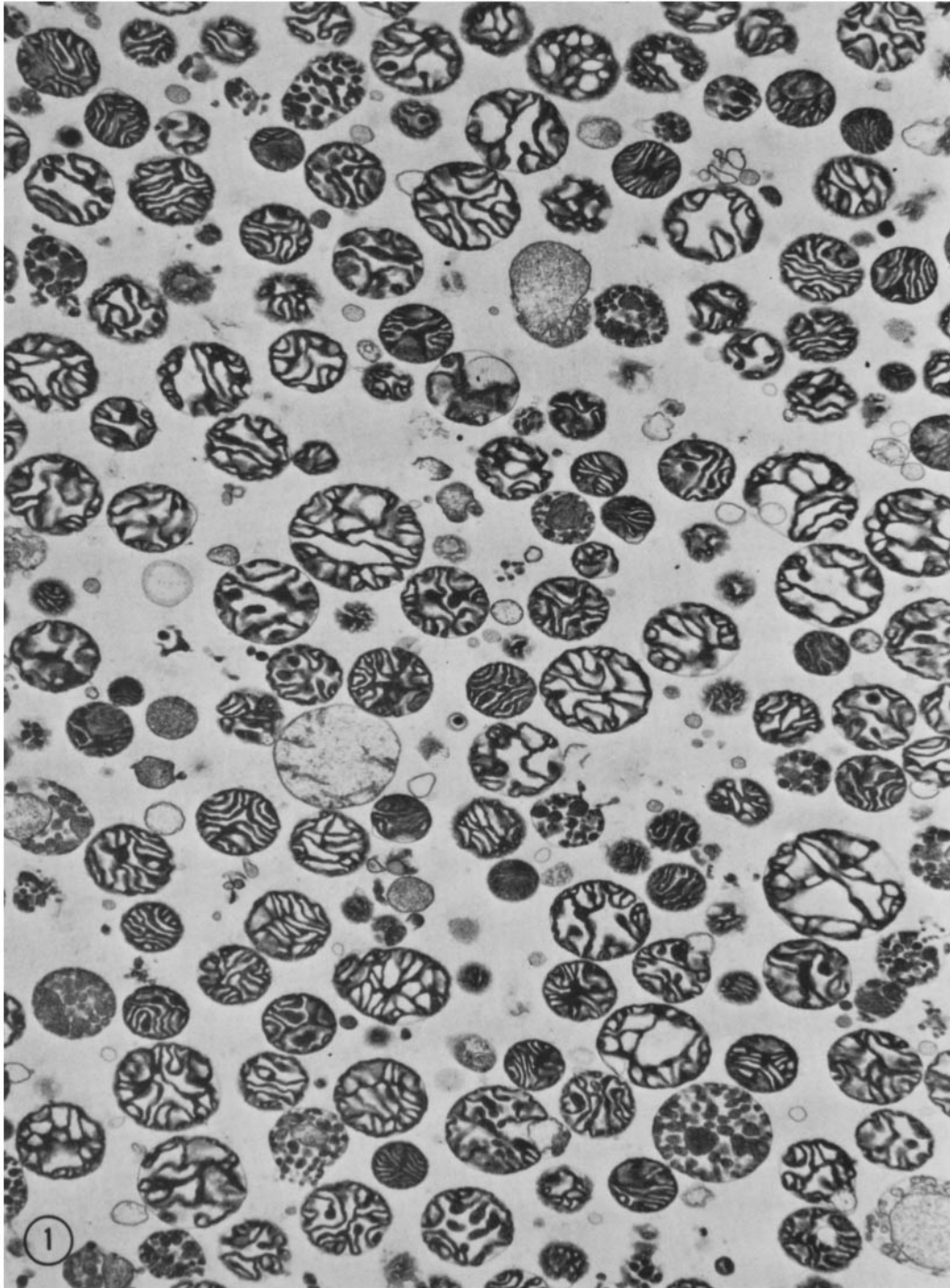


FIGURE 1 Survey micrograph showing the size and conformational state of isolated rat pancreas mitochondria.  $\times 12,000$ .

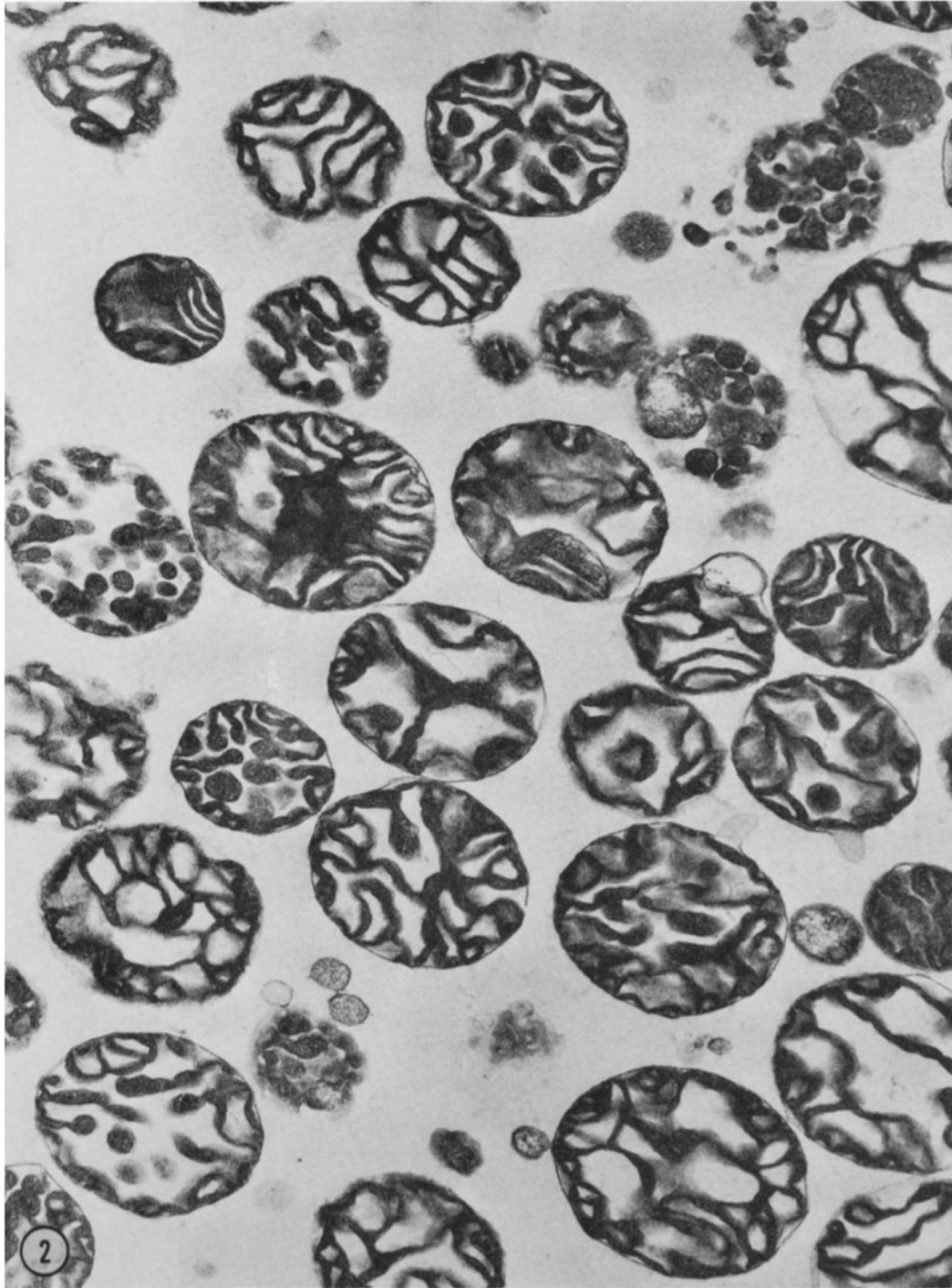


FIGURE 2 Electron micrograph of isolated rat pancreas mitochondria at higher magnification.  $\times 28,000$ .

TABLE I  
ATPase Activity of Isolated Rat Pancreas Mitochondria

Addition	Freshly isolated mitochondria (8)		Aged mitochondria (4)		Aged mitochondria in a medium containing BSA + Nupercaine (4)	
	a	b	a	b	a	b
Basal	19 ± 4*	13 ± 5	12 ± 3	13 ± 3	18 ± 4	14 ± 3
Mg <sup>2+</sup>	53 ± 7	33 ± 6	138 ± 9	110 ± 15	57 ± 7	41 ± 5
DNP	147 ± 13	272 ± 15	21 ± 5	103 ± 10	134 ± 19	259 ± 12

Freshly isolated mitochondria were used for ATPase assay. A part of the mitochondria was aged at 0°C for 6 h, in a medium containing mannitol-sucrose-Tris-EDTA as described, supplemented or not with 0.5% BSA + 0.4 mM Nupercaine. The basic medium for ATPase assay contained (0.2 ml final volume): 100 mM KCl, 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 5 mM ATP, and about 0.15 mg of mitochondrial protein. To this medium, Mg<sup>2+</sup> (5 mM), BSA (0.5%), or DNP (0.2 mM) was added when indicated. The results are expressed as nanomoles released phosphate per minute per milligram of protein at 37°C. Number of experiments is given in parentheses.

(a) Without BSA in the reaction medium, (b) with BSA (0.5% final concentration) in the reaction mixture.

\* Standard error of the mean.

mitochondrial ICDH (NAD- and NADP-linked enzyme) in pancreas, or to an NAD-specific enzyme role in the aerobic oxidation of isocitrate (21). The ratios of these activities in rat brain, skeletal muscle, heart, kidney, and liver mitochondria were found to be about 4.5, 1.1, 0.23, 0.24, and 0.47, respectively (22).

In conclusion, the described procedure for obtaining purified rat pancreas mitochondria is simple and reproducible, and it does not require special technical improvements for differential centrifugation. The structural and enzymatic properties of mitochondrial preparations depend on the following factors: (a) short homogenization time of the tissue, (b) homogenization in a small volume, and (c) the composition of the isolating media. The composition of the isolating media is of interest because sucrose (or mannitol) alone cannot replace the combined polyol media. EDTA below 0.3 mM would yield damaged mitochondria. The albumin (0.5%) protects mitochondria against swelling and provides an optimal viscosity for separation from zymogen granules. Smaller BSA concentrations (<0.2%) produced contaminated mitochondrial preparations; higher BSA concentrations (1%) aggregate the organelles.

For a correct estimation by electron microscopy of the functional state of isolated mitochondria, a suitable fixation procedure is a very important

condition. The best results were obtained by direct addition of glutaraldehyde dissolved in mannitol-sucrose-Tris medium to the mitochondrial suspension. By using a small amount of mitochondria (about 0.05 mg) we obtained thin layers of organelles after centrifugation, which were easily permeable to various substances and suitable for further morphological and histochemical preparation.

The maximal activity of the enzymes bound to the inner membrane or matrix space was obtained after Lubrol WX treatment of mitochondria. Other detergents (sodium deoxycholate, Triton-X-100) or decrease in medium tonicity were less effective in releasing "latent" mitochondrial enzymes.

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TABLE II  
Enzymatic Activity of Rat Pancreas and  
Liver Mitochondria

Enzyme	Rat pancreas	Rat liver
MAO		
Kynuramine	2.1	5.3
Benzylamine*	17.0	16.3
Cytochrome oxidase†	38	45
SDH	26	29
HBDH	3	110
MDH	1,780	3,250
	1,375§	2,920§
	744	2,930
ICDH	133¶	68¶
Fumarase	520	750

The activity of mitochondrial enzymes was measured after a previous solubilization of the organelles with the nonionic detergent Lubrol WX (0.5 mg/mg of protein). The reaction medium of 1 ml final volume contained 0.02–0.20 mg of mitochondrial protein. In some experiments mitochondria were disrupted with sodium deoxycholate (0.5 mg/mg of protein) or osmotically shocked, decreasing the medium tonicity to 5 mosmols/liter. The specific activity is expressed as nanomoles substrate transformed at 25°C per minute per milligram of protein. Number of experiments varies between three and eight.

\* Activity measured at 37°C.

† The specific activity is calculated from the first order reaction velocity constant ( $\text{min}^{-1} \times \text{mg protein}^{-1} \times \text{ml}$ ).

§ Mitochondria solubilized with Na deoxycholate.

|| Osmotically shocked mitochondria.

¶ With NADP as acceptor.

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