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PURIFICATION OF PIG HEART MITOCHONDRIAL MEMBRANES

ENZYMATIC AND MORPHOLOGICAL CHARACTERIZATION AS COMPARED TO MICROSOMES

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SUMMARY

1. Inner and outer membranes were purified from pig heart mitochondria using a procedure derived from Parsons' method (Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D. and Chance, B. (1967) Mitochondrial Structure and Compartmentation (Quagliariello, E., Papa, S., Slater, E. C. and Tager, J. J., eds), pp. 29–70, Adriatica Editrice, Bari including swelling and sucrose-gradient fractionation; an additional step made it possible to isolate microsomes.

2. Purification of the membranes was followed by the localization of marker enzymes by De Duve plots and by electron microscopy.

3. Outer membranes were purified 13-fold as shown by monoamine oxidase activity. Inner membranes were purified 5-fold as estimated by rotenone-sensitive NADH-cytochrome *c* reductase. No glucose-6-phosphatase or monoamine oxidase or cytochrome oxidase could be detected in microsomes.

4. Our results are in favour of, but do not prove, a dual localization of NADPH-cytochrome *c* reductase in microsomes and outer membranes.

INTRODUCTION

The separation of the inner and outer membranes of liver mitochondria seems to be relatively easy to perform either by high-amplitude swelling in the presence of inorganic phosphate [1–3] or by the use of digitonin [3–5].

The problem with heart mitochondria seems far more difficult, perhaps because of the very small surface of outer membranes as compared to the great number of cristae. Several attempts have been made to obtain heart mitochondrial membranes from beef [6–9], guinea-pig [10] and rabbit [11], mainly to localize enzymatic activities.

According to Jacobus and Lehninger [12] at the beginning of 1973, "no satisfactory method was yet available for the unambigous and quantitative separation of

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the outer and inner membranes of heart mitochondria". Recently Scholte [13] has set up a method modified from that of Sottocasa et al. [2], to achieve a rapid separation of the inner and outer membranes from rat heart mitochondria on a small scale. Their technique included the use of a proteinase.

The purpose of the present work was to obtain inner membranes as pure and intact as possible from pig heart mitochondria in view of a further study of thiol-bearing proteins possibly implied in the energization of the inner membrane and energy conservation [14] and to elucidate the role of an isolated proteolipid in relation to glutamate transport [15]. In this case the use of a proteinase should be absolutely avoided. Pure inner and outer membranes were obtained by the described method.

METHODS

Pig hearts obtained from the slaughter house and brought back to the laboratory packed in ice were used 30 min after the electrocution of the animals. Mitochondria were isolated according to Crane et al. [16] and washed, tested for respiratory control ratios, protein concentration and ADP/O, as previously [17].

Marker enzymes

The following markers were used to assess the purity of the fractions: monoamine oxidase (EC 1.4.3.4), rotenone-sensitive NADH-cytochrome *c* reductase and malate dehydrogenase (EC 1.1.1.37) for, respectively, outer membrane, inner membrane and matrix. Glucose-6-phosphatase (EC 3.1.3.9) and NADPH-cytochrome *c* reductase are reputed microsomal markers.

In each fraction the following activities have been tested: monoamine oxidase was assayed according to Schnaitman and Greenawalt [18] after a modification of the spectrophotometric procedure of Tabor et al. [19] in the presence of 0.025 % Lubrol WX (final) or 0.166 % (final) Triton X-100 using benzylamine as substrate. Cytochrome *c* oxidase (EC 1.9.3.1) was tested polarographically according to Sottocasa et al. [2]. A 5 min treatment by 0.025 % (final)Lubrol WX at 30 °C abolished the latency of the enzyme.

NADH-, NADPH-, and succinate-cytochrome *c* reductase were assayed spectrophotometrically by following the reduction of added cytochrome *c*, at 550 nm in the presence of KCN, in the presence or not of 4 μM rotenone, according to Sottocasa et al. [2].

Malate dehydrogenase was assayed according to Ochoa [20] by following the decrease in adsorbance of added NADH at 340 nm, in the presence of 4 μM rotenone and 80 μM Na_2S to prevent reoxidation of NADH by the respiratory chain and to inhibit NADH oxidase activity. To abolish enzyme latency, the fractions were pretreated with 0.015 % (final) Triton X-100 for 5 min at 28 °C.

Kynurenine 3-monooxygenase was tested by the method of Hayashi [21].

D-glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of De Duve et al. [22]; inorganic phosphate released during 15 min incubation was determined as described by Bartlett [23]. Protein in fractions was estimated according to Lowry et al. [24]. Electron microscopy was performed at the Centre de Microscopie Electronique appliquée à la Biologie, de l'Université Claude Bernard de Lyon, by Claudine

Vitorelli using HU 11 A and Philips EM 300 microscopes.

Before the thin sections were observed, the following treatments were applied: glutaraldehyde fixation, osmium tetroxide postfixation, in sodium cacodylate–HCl buffer, pH 7.4. Ultrathin sections were contrasted successively by uranyl acetate dissolved in methanol and lead citrate. Negative staining was performed with phosphotungstic acid + NaOH. The samples were fixed or not by osmium tetroxide.

RESULTS

Isolation and purification procedures of the fractions

Mitochondria were prepared from 100 g homogenized fresh pig heart in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 and washed twice in the same medium before fragmentation.

The fractionation procedure of mitochondria is described in Fig. 1 and was

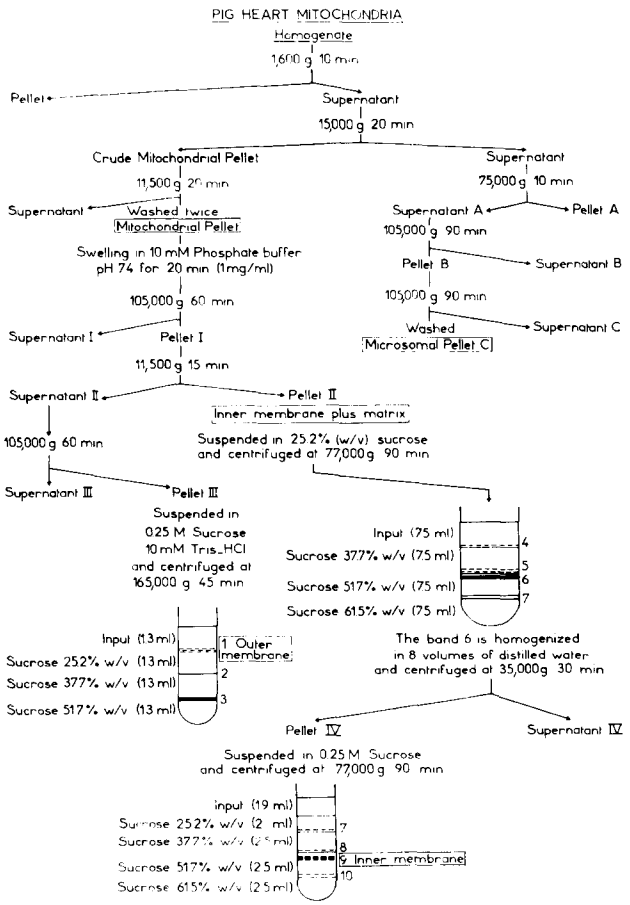


Fig. 1. Isolation procedure of pig heart mitochondria membranes and microsomes. All sucrose density gradients are buffered with 10 mM Tris–HCl pH 7.4. Layers 1 (outer membrane), 9 (inner membrane) and Pellet C (crude microsomes) are diluted in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 and spun down as pellets, then they are homogenized in the same medium prior to the enzymatic assay.

derived from the method applied by Parsons et al. [1] to guinea pig liver mitochondria. Applied accelerations were specifically determined since pig heart mitochondrial membranes seemed lighter than guinea pig liver membranes.

The washed mitochondrial pellet (400–500 mg protein) was homogenized in a Potter with a loosely fitting pestle in 10 mM potassium phosphate pH 7.4, 0 °C, then diluted in the same medium (1 mg protein/ml) and allowed to swell for 20 min. Then the suspension was centrifuged at 105 000 × *g* for 60 minutes to eliminate the intermembrane enzymes (Supernatant I). Pellet I, which contained outer and inner membranes and matrix, was resuspended in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 and spun down at 11 500 × *g* for 15 min in order to separate the outer membranes (Supernatant II) from the inner membranes and matrix (Pellet II).

Supernatant II was centrifuged for 60 min at 105 000 × *g* to recover most of the outer membranes (Pellet III) while Supernatant III was discarded since it contained mainly matrix enzymes as tested by malate dehydrogenase activity.

Pellet III was resuspended in 5 ml, 0.25 M sucrose, 10 mM Tris–HCl and purified by centrifugation through a discontinuous sucrose density gradient (25.2 %, 37.7 %, 51.7 % w/v) in an SW 39 L rotor-Spinco centrifuge (38 000 rev./min, 165 000 × *g*, 90 min). The outer membranes were recovered, in the top layer, Fraction 1 between *d* 1.093–1.130, free of inner membranes. Fractions 2 and 3 containing, respectively, outer membranes and mitochondrial fragments and inner membranes were discarded.

Pellet II containing mitoplasts was suspended in 22 ml, 25.2 % sucrose and passed twice through discontinuous sucrose density gradients to purify the inner membranes (sucrose 25.2 %, 37.7 %, 51.7 % and 61.5 % w/v). After a first passage on the gradient (SW 25-1 rotor, Spinco centrifuge at 23 500 rev./min, 77 000 × *g* for 90 min) Band 6, which contained the major part of the inner membranes, was homogenized in 8 vol. of distilled water to break any residual mitoplasts, then spun down at 35 000 × *g* for 30 min; Supernatant IV containing matrix enzymes was discarded and Pellet IV was passed through the same discontinuous sucrose gradient (41 Ti rotor, Spinco centrifuge at 21 000 rev./min, 77 000 × *g* for 90 min). The final Layer 9 was recovered and contained pure inner membranes.

All sucrose solutions were buffered with 10 mM Tris–HCl and all operations were conducted at 0–4 °C.

Layers 1 (outer membrane) and 9 (inner membrane) were eventually diluted in 0.25 M sucrose, 10 mM Tris–HCl and spun down to recover the membranes as pellets prior to the assays.

The fractionation procedure (Fig. 1) was planned to allow the preparation of microsomes. The post-mitochondrial supernatant was centrifuged at 75 000 × *g* for 10 min and the pellet containing small mitochondria and fragments was discarded. Supernatant A was centrifuged at 105 000 × *g* for 90 min and Pellet B was recovered and recentrifuged in the same way to wash microsomes. The final microsomal pellet was resuspended in 0.25 M sucrose, 10 mM Tris–HCl at pH 7.4 for enzymatic assays and electron microscopy.

Validity of the isolation procedures as supported by the De Duve's plot [22]

To adjust the fractionation procedure so that membranes as pure as possible could be obtained, every important step was checked by plotting the marker enzyme activities according to De Duve [22].

Outer membranes. In Fig. 2 we see that during the first purification steps (swelling and differential centrifugations) the monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* reductase activities increased significantly only in Fraction 2 = Pellet III by 12- and 8-fold, respectively. Therefore, in order to purify the outer mem-

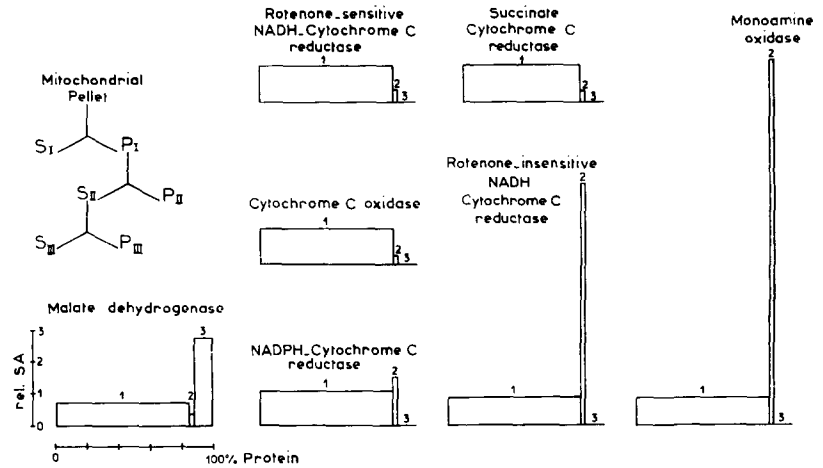


Fig. 2. The partition of the marker enzymes in the first steps of mitochondrial membrane isolation. 1, Pellet II = inner membrane plus matrix; 2. Pellet III = crude outer membrane; 3, Supernatant II plus Supernatant III = soluble fraction. Rel. SA, relative specific activity.

branes, only Pellet III was passed on a sucrose density gradient. We can see in Fig. 3B that Layer 1, obtained in the sucrose density gradient centrifugation of Pellet III, which contained most of mitochondrial monoamine oxidase activity (see Table I)

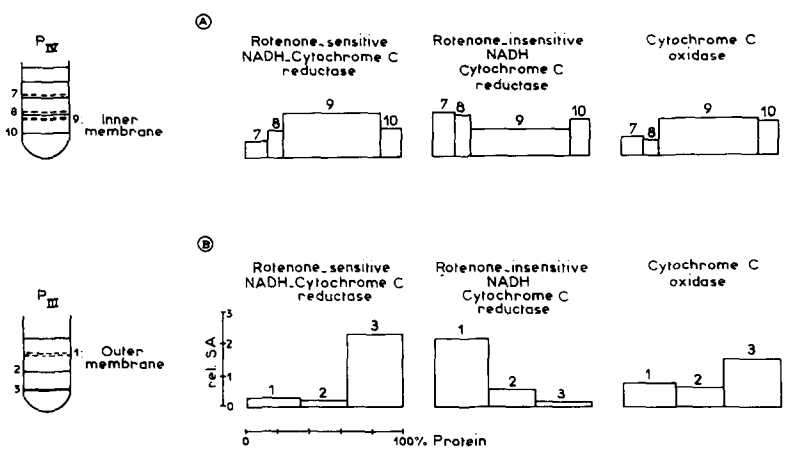


Fig. 3. The partition of marker enzymes. A, in the second sucrose density gradient for purified inner membrane isolation: 7, Layer 7; 8, Layer 8; 9, Layer 9; 10, Layer 10. B, in the sucrose density gradient for purified outer membrane isolation: 1, Layer 1; 2, Layer 2; 3, Layer 3. Rel. SA, relative specific activity.

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN MITOCHONDRIAL AND MICROSOMAL MEMBRANE PREPARATIONS

Specific activities expressed in nmoles. · min^{-1} · mg^{-1} protein ± S.E.; in parentheses, number of experiments.

Fraction	Rotenone-insensitive NADH-cytochrome *c* reductase	Rotenone-sensitive NADH-cytochrome *c* reductase	Succinate-cytochrome *c* reductase	NADPH-cytochrome *c* reductase	Monoamine oxidase★	Malate dehydrogenase★	Cytochrome *c* oxidase★
Mitochondria	75 ± 5.4 (16)	57.2 ± 4.8 (15)	89.6 ± 8.1 (7)	19.6 ± 2.2 (12)	0.97 ± 0.14 (11)	11 650 ± 2350 (2)	1100 ± 380 (3)
Inner membrane plus matrix	49.2 ± 5.1 (10)	193.2 ± 12.8 (8)	151 ± 15 (7)	20.4 ± 3.7 (9)	0.47 ± 0.17 (6)	10 200 ± 2800 (2)	1730 ± 720 (2)
Inner membrane	39 ± 10 (7)	265.5 ± 39.1 (7)	242 ± 40 (6)	22.4 ± 6 (6)	0.33 ± 0.23 (3)	6 870 ± 970 (2)	2930 ± 430 (2)
Outer membrane	382 ± 36 (9)	17.6 ± 7.9 (9)	18.8 ± 10.8 (6)	85.7 ± 9.8 (9)	12.7 ± 2.6 (7)	171 (1)	225 ± 111 (2)
Microsomes	103 ± 10.3 (4)	29.2 ± 7.8 (4)	< 0.1 (1)	87 ± 8.3 (5)	0.38 ± 0.1 (4)	—	< 0.1 (1)
Ratio							
$\frac{\text{Outer membrane}}{\text{Mitochondria}}$	5.1	0.3	0.21	4.3	13.1	0.015	0.22
$\frac{\text{Inner membrane}}{\text{Mitochondria}}$	0.52	4.6	2.7	1.1	0.34	0.59	2.65
$\frac{\text{Outer membrane}}{\text{Microsomes}}$	3.7	0.6	—	1	33.5	—	—

★ Monoamine oxidase, malate dehydrogenase and cytochrome *c* oxidase activities were measured in the presence of detergents.

also exhibits the highest specific activity for rotenone-insensitive NADH-cytochrome *c* reductase (another marker of the outer membranes [2]) while it has very low rotenone-sensitive NADH-cytochrome *c* reductase and cytochrome oxidase activities. Layers 2 and 3 on the gradient were discarded in view of their low monoamine oxidase activity.

Starting from 500 mg mitochondrial protein, about 2–3 mg protein were recovered in the purest outer membrane fraction.

Inner membranes. We see in Fig. 2 that the three main marker activities of inner membranes, cytochrome *c* oxidase, rotenone-sensitive NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase, increased similarly during the first steps of purification.

Fraction 1 = Pellet II contained most of the inner membranes and matrix. Two sucrose density gradients were applied to eliminate the matrix enzymes. Band 6 obtained in the fractionation procedure still contained significant amounts of matrix enzymes. Fig. 3A shows that the final protein Band 9 is the richest in cytochrome oxidase and rotenone-sensitive NADH-cytochrome *c* reductase (see also Table 1).

During the entire fractionation procedure, malate dehydrogenase, a reputed matrix enzyme, was widely released in the soluble fractions (supernatant Fractions I, III and IV).

Microsomes. Pig heart microsomal fractions exhibited no glucose-6-phosphatase activity whatever the preparation procedure used and, therefore, this enzyme could not be used as a marker.

Fig. 4 shows that Pellet B (crude microsomes) exhibited the highest NADPH-cytochrome *c* reductase (rotenone insensitive) and rotenone-insensitive NADH-cytochrome *c* reductase activities, while almost no rotenone-sensitive NADH-cytochrome *c* reductase was present. Routinely, one washing was applied to this crude microsomal fraction before further analysis.

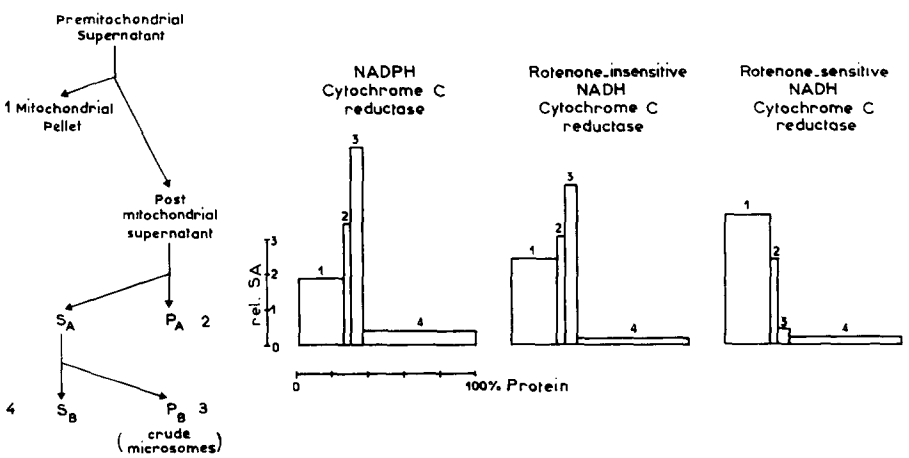


Fig. 4. The partition of the marker enzyme in the first steps of the microsome isolation. 1, mitochondrial Pellet; 2, Pellet A = (mitochondria plus microsomes); 3, Pellet B = crude microsomes; 4, Supernatant B = post-microsomal supernatant. Rel. SA, relative specific activity.

Enzymatic specific activities of the fractions. Table I summarizes the specific activities of the various markers, in the fractions. According to the different markers, the inner membranes were enriched 3- to 5-fold and comparison with the markers of other fractions indicated that there was hardly any contamination. It is striking, however, that significant malate dehydrogenase activity remained associated with the inner membrane fraction in spite of three ordinary centrifugations and two passages on discontinuous sucrose density gradients.

Table II shows that Lubrol WX significantly increased the cytochrome oxidase activity in inner membrane, while it inhibited succinate- and NAD(P)H-cytochrome

TABLE II

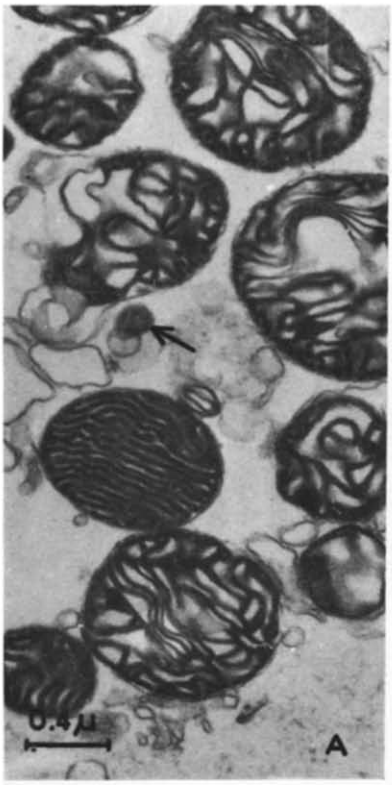
EFFECT OF DETERGENTS

Specific activities expressed in μmoles. $\cdot$ min^{-1} $\cdot$ mg^{-1} protein $\pm$S.E.; in parentheses: number of experiments.

	Mitochondria	Mitoplast	Inner membrane	Outer membrane
A. Cytochrome *c* oxidase				
− Lubrol	0.395 $\pm$ 0.073 (7)	0.913 $\pm$ 0.341 (3)	1.236 $\pm$ 0.068 (3)	—
+ Lubrol	1.10 $\pm$ 0.38 (3)	1.73 $\pm$ 0.72 (2)	2.93 $\pm$ 0.43 (2)	0.225 $\pm$ 0.111 (2)
B. Malate dehydrogenase				
− Triton	2.65 $\pm$ 1.35 (2)	4.3 $\pm$ 0.7 (2)	3.60 $\pm$ 0.01 (2)	—
+ Triton	11.65 $\pm$ 2.35 (2)	10.2 $\pm$ 2.8 (2)	6.87 $\pm$ 0.97 (2)	0.171 (1)

c reductase activities, as observed by Schnaitman [18]. However, the treatment by Triton X-100 increased 2-fold the malate dehydrogenase activity of the inner membrane fraction as it does for cytochrome oxidase.

Fig. 5. Electron micrography of pig heart mitochondrial and microsomal membranes. A. Thin-section of pig heart mitochondria used in preparing purified mitochondrial membrane fractions. A few contaminating particles still unidentified (→) are present. Note the great number of cristae. Magnification × 25 000. B. Thin-section of pig heart mitochondria inner membrane. It consists of intact inner membrane ghosts which have no attached outer membrane. Magnification × 16 000. C. Thin-section of purified pig heart outer membrane (Layer 1). The preparation consists of rather uniform spherical vesicles. Very little rough-surfaced endoplasmic reticulum is present (→), but smooth-surfaced vesicles cannot be distinguished in thin section from outer membranes vesicles. Magnification 38 400. D. Negatively stained (non-fixed) preparation of microsomes (Pellet C). Most of the pieces show a thick white border. Magnification × 35 500. E. Negatively stained (non-fixed) purified pig heart mitochondria outer membrane. We can see a similar white border to that seen with microsomes but outer membrane vesicles are much larger than microsomes. The pieces marked (→) are probably 90-Å inner membrane particles. Magnification × 35 500. F. Negatively stained purified pig heart mitochondria outer membrane fixed by osmium tetroxide. The outer membrane has a characteristic "folded bag" appearance. Some pieces of endoplasmic reticulum (→) have survived the fixation treatment. Magnification × 63 800.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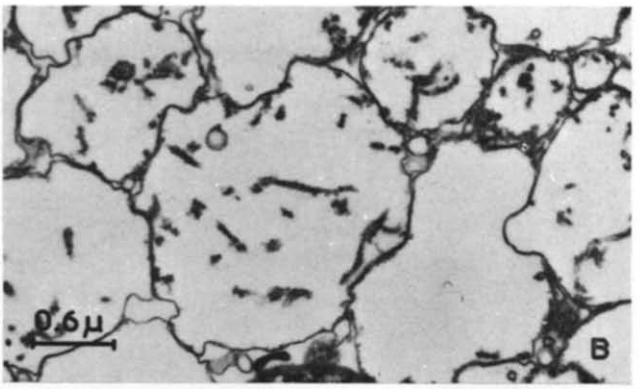
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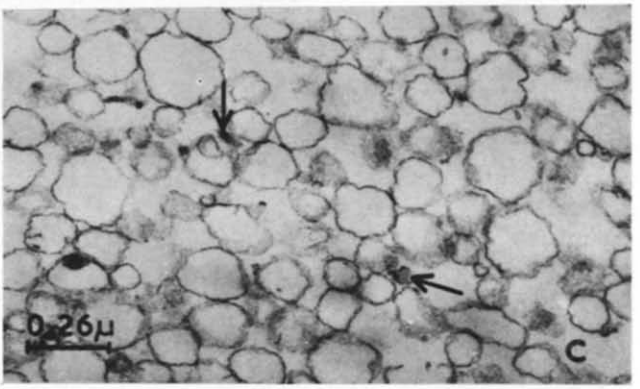
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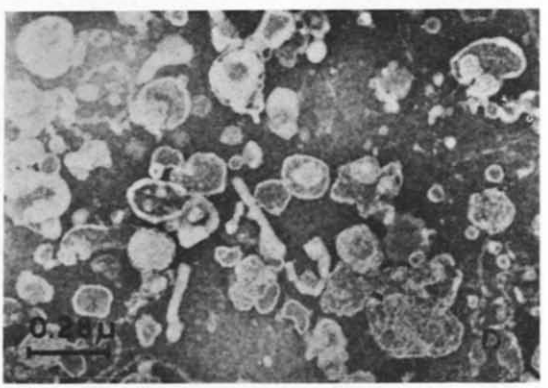
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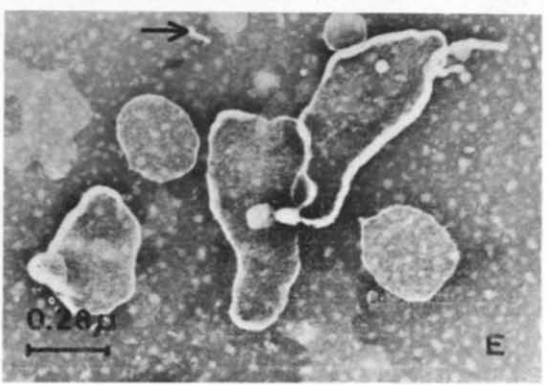
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The outer membranes have been purified 13-fold as shown by the monoamine oxidase activity (Table 1). The calculated ratios indicate less than 8 % contamination by inner membranes. We see that NADPH-cytochrome *c* reductase which is supposed to be a microsomal marker [13] had the same specific activity both in microsomal and outer membrane fractions.

Contrary to this, the microsomes did not show any significant activity of the inner and outer mitochondrial membrane marker enzymes (succinate-cytochrome *c* reductase, cytochrome oxidase or monoamine oxidase).

Electron microscopy

The purification and identification of the membrane fractions were followed by electron microscopy. Fig. 5 shows that the electronmicrographs corroborate the results obtained with marker enzymes.

We see in Fig. 5C (fixed thin-section) and Fig. 5F (fixed negative staining) that the outer membrane fractions exhibited some microsomal contamination but were essentially composed of outer membranes. If one compares the microsomes (Fig. 5D) and outer membranes (Fig. 5E) at the same magnification ($\times$35 500) their structures appear extremely different.

Taking into account the magnifications we see that the unfolded inner membrane diameters are about three times that of the whole mitochondria, while the diameter of the outer membrane vesicles appears to be only about a quarter.

DISCUSSION

Our major problem was to obtain inner membranes as pure as possible. From the data given by marker enzymes and electron microscopy we can conclude that our objective has been reached. The inner membranes were purified 3- to 5-fold and they contained only a minor amount of contamination. The specific activities of their marker enzymes were comparable with those described in the literature for mitochondria from other sources [1–11, 13, 25, 26]. The possibility of an association of malate dehydrogenase with inner membrane cannot be excluded since after repeated fractionation and purification procedures, the specific activity of this enzyme is high in the inner membranes and treatment by detergents increased both the cytochrome oxidase and malate dehydrogenase activities in these membranes by the same amount. Such an association could be possible in view of the interaction of malate dehydrogenase with phospholipid membranes [27] and since the matrix in heart mitochondria is packed with cristae (Fig. 5A).

The outer membranes were purified 13-fold which is comparable to the best preparations of rat liver mitochondria outer membranes [2, 3]. Recently, Hayashi and Capaldi [9] obtained a preparation from beef heart 28-times enriched in monoamine oxidase but only 4.2-times in rotenone-insensitive NADH-cytochrome *c* reductase, however, as it was pointed out, this last enzyme is located in the outer membrane as well as in the sarcoplasmic reticulum [2, 13]. Although the outer membranes amount to less than 5 % of the inner membranes in pig heart mitochondria, less than 8 % contamination by the marker enzymes of the inner membranes was observed. Considering the outer membrane yield, any additional purification step would result in no outer membrane at all. Some microsomal contamination was present in the outer

membranes. The high activity of NADPH-cytochrome *c* reductase, a reputed microsomal marker, could suggest a high microsomal contamination. The electron micrographs did not show a high contamination by microsomes. The NADPH-cytochrome *c* reductase specific activity was exactly the same in the outer membranes and microsomes, giving a ratio of outer membranes/microsomes of 1 for this activity. If the amounts of microsomes and outer membranes in the original heart homogenate were about the same, such a ratio would absolutely exclude the possibility that the NADPH-cytochrome *C* reductase of the outer membrane preparation could be due to contamination, either by whole microsomes or by some enzymatic activity released from the microsomes during the fractionation procedure. It is unfortunate that pig heart microsomes did not exhibit any glucose-6-phosphatase whatever the treatment applied (none of the other heart fractions contained such an activity, as expected); this agrees with Scholte's [13] results. To try to evaluate further the microsome contamination in our preparations, we tried repeatedly to prepare only heavy mitochondria, to pass them on a sucrose density gradient to purify them prior to any other fractionations. The NADPH-cytochrome *c* reductase activity remained the same before and after purification. In the same way the purest outer membrane fraction (Layer 1) re-run on a discontinuous sucrose density gradient (sucrose 12.5 %, 25.2 %, 37.7 %) to eliminate microsomes, exhibited the same NADPH-cytochrome *c* reductase activity. Therefore, one could be tempted to conclude that this activity in pig heart belongs to outer membrane as well as to microsomes. However, owing to the very small amount of outer membranes, and thus the small fraction of the total activity of NADPH-cytochrome *c* reductase of the whole heart homogenate recovered with outer membranes, a further investigation will be necessary to support such a conclusion. Brunner and Bygrave [28] have questioned the validity of this marker even in rat liver fractions. The data reported by Van Tol [29] in fact do not agree with his own conclusions.

The homogeneity of outer membrane vesicles and their size as compared to whole mitochondria suggest that during the swelling procedures the outer membranes were broken at several points and spontaneously reformed vesicles. This might be in relation to some junction points of outer membrane with inner membrane as pointed out by Scholte [13].

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