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Functional and Biogenetical Heterogeneity of the Inner Membrane of Rat-Liver Mitochondria

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Rat liver mitochondria were fragmented by a combined technique of swelling, shrinking, and sonication. Fragments of inner membrane were separated by density gradient centrifugation. They differed in several respects: electronmicroscopic appearance, phospholipid and cytochrome contents, electrophoretic behaviour of proteins and enzymatic activities.

Three types of inner membrane fractions were isolated. The first type is characterized by a high activity of metal chelatase, low activities of succinate-cytochrome *c* reductase and of glycerolphosphate dehydrogenase, as well as by a high phospholipid content and low contents of cytochromes aa_3 and *b*.

The second type displays maximal activities of glycerolphosphate dehydrogenase and metal chelatase, but contains relatively little cytochromes and has low succinate-cytochrome *c* reductase activity.

The third type exhibits highest succinate-cytochrome *c* reductase activity, a high metal chelatase activity and highest cytochrome contents. However, this fraction was low in both glycerolphosphate dehydrogenase activity and phospholipid content. This fraction was also richest in the following enzyme activities: cytochrome oxidase, oligomycin-sensitive ATPase, proline oxidase, 3-hydroxybutyrate dehydrogenase and rotenone-sensitive NADH-cytochrome *c* reductase.

Amino acid incorporation *in vitro* and *in vivo* in the presence of cycloheximide occurs predominantly into inner membrane fractions from the second type.

These data suggest that the inner membrane is composed of differently organized parts, and that polypeptides synthesized by mitochondrial ribosomes are integrated into specific parts of the inner membrane.

Polypeptide chains synthesized by the mitochondrial ribosomes are integrated into the inner but not outer mitochondrial membrane [1,2]. However, it was observed that the incorporation *in vitro* of radioactive amino acids into mitochondrial membranes did not follow the distribution of inner membrane marker enzymes such as succinate-cytochrome *c* reductase [3]. This raises the question of whether the integration of newly synthesized polypeptides is subject to a random mechanism or takes place at specific regions along the membrane. This problem again involves the question of whether the mitochondrial inner membrane is uniform in structure and function throughout its whole extension, or whether it exhibits a degree of differentiation.

Although this problem has been attacked on a morphological basis [4,5], no biochemical data are as yet available. Therefore, a study was made to subfractionate the inner membrane of rat liver mitochondria, essentially with the technique of swelling, shrinking and sonication, combined with sucrose-density-gradient centrifugation. A heterogeneous structure of the inner membrane should be reflected in the production of fragments, which differ with respect to their enzyme contents, protein and lipid patterns as well as their morphological appearance.

Enzymes. Cytochrome aa_3 or cytochrome oxidase or ferrocytochrome *c*: oxygen oxidoreductase (EC 1.9.3.1); glycerolphosphate dehydrogenase or L-glycerol-3-phosphate: (acceptor) oxidoreductase (EC 1.1.99.5); D-3-hydroxybutyrate dehydrogenase or D-3-hydroxybutyrate: NAD oxidoreductase (EC 1.1.1.30); monoamine oxidase or monoamine: oxygen oxidoreductase (deaminating) (EC 1.4.3.4); metal chelatase or protohaem ferro-lyase (EC 4.99.1.1); malate dehydrogenase or L-malate: NAD oxidoreductase (EC 1.1.1.37).

METHODS

Preparation of Mitochondria and Microsomes

Adult male albino rats (about 200 g) were starved for 15 h prior to sacrifice. Liver mitochondria were prepared in a medium containing 0.25 M sucrose, 2 mM EDTA, 10 mM triethanolamine buffer, pH 7.3

according to the method of Schneider [6]. They were washed by three successive low and high spin centrifugations (10 min, $700\times g$; 10 min, $7000\times g$). For separation of microsomes the supernatant from the first step sedimenting mitochondria was centrifuged for 30 min at $18000\times g$. Microsomes in the supernatant were sedimented by centrifugation for 60 min at $105000\times g$, and subsequently suspended in 0.1 M phosphate buffer, pH 7.6.

Subfractionation of Mitochondria

A modified combined technique of swelling, shrinking and sonication as described by Parsons *et al.* [7,8] and Sottocasa *et al.* [9] was applied to subfractionate mitochondria. The final mitochondrial pellet was carefully resuspended in 60 ml of 10 mM triethanolamine buffer, pH 7.3, containing 2 mM EDTA, using a glass homogenizer with a teflon pestle. The suspension was allowed to stand for 10 min. Then 20 ml of a medium containing 1.75 M sucrose, 8 mM ATP, pH 7.6, was added. During the shrinking period (10 min) the mitochondria were gently homogenized. The swollen and shrunken particles were sonified in 20 ml aliquots for 2×5 sec with a Branson sonifier (Model S 75, micro-tip, 3.5 A output).

The sonified suspensions were centrifuged for 20 min at $20000\times g$ and supernatants discarded. The pellets were resuspended in isolation medium and centrifuged for 10 min at $1250\times g$. The resulting pellet accounts for about 50% of total mitochondrial protein. On the basis of electronmicroscopic and enzymatic data this fraction essentially consists of mitochondria devoid of outer membrane. It was concluded that it does not represent fragments of inner membrane and it was not used in further studies. The supernatant was centrifuged for 20 min at $20000\times g$.

The resulting pellet was resuspended in 5 ml of isolation medium (about 5 mg protein/ml) and layered above a linear sucrose-density gradient (gradient A) containing 50 ml. The densities varied between 1.13 and 1.25 g/ml. Linearity of gradients was established by controlling the refractive indices. The gradient was centrifuged in a Beckman Spinco apparatus (rotor 25.2) for 60 min at $75000\times g$. The gradient was subdivided into 10 fractions of 5 ml (the pellet is referred to as fraction 11). Fractions 1 to 10 were diluted to a sucrose concentration of 0.25 M; fraction 11 was taken up in isolation medium. The suspensions were centrifuged for 30 min at $165000\times g$, using a Spinco rotor 50 Ti. The pellets were then separately suspended in 11 ml 0.1 M potassium phosphate buffer, pH 7.6, and recentrifuged for 30 min at $165000\times g$. The resulting pellets were resuspended in the same buffer, to give a final protein concentration of about 4 mg/ml.

In order to remove soluble proteins, the fractions were sonified 8×15 sec in 5 ml phosphate buffer (0.1 M, pH 7.6), using the Branson sonifier (Model S 75, micro-tip, 3.5 A output). Then they were centrifuged for 90 min at $165000\times g$. The pellets were suspended in 0.5 to 1.0 ml phosphate buffer with sonification (2×10 sec at minimum output).

Three mitochondrial subfractions (1, 3 and 6) were subjected to further sucrose-density-gradient centrifugation. The three pellets obtained by centrifugation of the fractions from gradient A were suspended in 0.5 ml isolation medium. They were layered above linear sucrose gradients (containing 12 ml) with the following densities: fraction 1, 1.098 to 1.161 g/ml; fractions 3, 1.132 to 1.182 g/ml; fraction 6, 1.172 to 1.225 g/ml. After centrifugation for 60 min at $220000\times g$ in a Spinco SW 41 rotor, the gradients were subdivided into 9 equal fractions. These were then diluted to 11 ml with isolation medium and centrifuged at $165000\times g$ for 90 min. Soluble proteins were removed as described above.

All procedures were carried out at 0—4 °C.

Enzyme and Cytochrome Determinations

NADH-, NADPH-, succinate-cytochrome *c* reductase and mitochondrial glycerolphosphate dehydrogenase were measured by following the reduction of cytochrome *c* at 550 nm. The assay mixtures contained: 100 mM phosphate buffer, pH 7.6, 5 mM EDTA, 1 mM KCN, 0.08 mM cytochrome *c* and appropriate substrate: NADH (0.1 mM), NADPH (0.1 mM), succinate (25 mM), L-glycerolphosphate (20 mM). Rotenone sensitivity of NADH-cytochrome *c* reductase was tested in the presence of 2 μM inhibitor. In glycerolphosphate dehydrogenase assays, EDTA was replaced by 10 mM $MgCl_2$. Tests for succinate-cytochrome *c* reductase and glycerolphosphate dehydrogenase were carried out with several concentrations of phenazine methosulphate (standard concentration 0.1 mM).

Metal chelatase was determined in two different assay systems. In the first system the formation of heme from ferrous ion and protoporphyrin IX was measured under a nitrogen atmosphere [10]. In the second system the aerobic formation of cobalt · protoporphyrin IX complex was recorded at 433 nm and 37 °C [11]. Sample was first incubated for 5 min in 0.1 M phosphate buffer, pH 8.0, containing 0.5% Tween 80 and 50 μM protoporphyrin IX. The reaction was then started by addition of 12 μM Co^{2+}. The molar absorption coefficient at 433 nm was calculated by correlating the increase of absorbance at 433 nm with a simultaneous determination of residual protoporphyrin IX in the cuvette (assayed by removing aliquots into 1.5 N HCl and measuring the absorption of the Soret band). A value for $\Delta\varepsilon_{433\,nm}$ of 29.9 $mM^{-1}cm^{-1}$ was estimated. In a second

approach absolute spectra of protoporphyrin IX and cobalt · protoporphyrin complex (generously supplied by Dr. M. Schmidt, I. Medizinische Klinik, Universität München) were recorded under reaction conditions. The absorption coefficients obtained by the two different methods were in good agreement.

Oligomycin-sensitive ATPase was determined in the presence of an ATP-regenerating system, at pH 8.0, essentially as described by Pullmann *et al.* [12]. Oligomycin concentrations of 25 nmol per mg protein were employed. Reaction temperature was 30 °C.

Monoamine oxidase was measured according to the method of Tabor *et al.* [13]. Oxidation of benzylamine (5 mM) to benzaldehyde was recorded at 250 nm ($\varepsilon_{250\,nm} = 13.4\ mM^{-1}cm^{-1}$) in phosphate buffer, pH 7.6.

Proline oxidase was determined by the method described by Strecker [14, 15]. The assay mixture contained 100 mM phosphate buffer, pH 7.6, 20 mM L-proline, 1 μM cytochrome *c*, when indicated, and 0.4 to 0.8 mg protein per ml. The reaction temperature was 37 °C.

D-3-Hydroxybutyrate dehydrogenase was measured as described by Gotterer [16], including 1 mM KCN. The assays were run in the direction of NAD reduction with 3-hydroxybutyrate as substrate.

Malate dehydrogenase was determined according to Delbrück *et al.* [17].

Cytochrome oxidase was measured spectrophotometrically by following the decrease of absorbance at 550 nm of ferrocytochrome *c* (0.1 mM) in Tris-sulfate buffer, pH 7.4.

All enzyme reactions were started by the addition of substrate. If not otherwise stated, assay temperature was 25 °C.

Cytochrome aa_3, *b* and b_5 contents were calculated from difference spectra (reduced minus oxidized) performed with a Beckman DK 2A recording spectrophotometer, using the following molar absorption coefficients: Cytochrome aa_3, $\Delta\varepsilon_{605\,nm} = 24\ mM^{-1}\,cm^{-1}$ [18]; cytochrome *b*, $\Delta\varepsilon_{561\,nm} = 16\ mM^{-1}\,cm^{-1}$ [19]; cytochrome b_5, $\Delta\varepsilon_{424-405\,nm} = 160\ mM^{-1}\,cm^{-1}$ [20]. Cytochrome P-450 was estimated according to the procedure described by Omura and Sato [21].

Phospholipid and Protein Determinations

Lipids from intact mitochondria and submitochondrial fractions were extracted with chloroform—methanol (2:1, by vol.). Non-lipid phosphate was removed from extracts by partition according to the method of Folch *et al.* [22]. Lipid phosphorus was determined by a modified procedure of Fiske and SubbaRow [23].

Protein was measured by a modification of the biuret method [24].

Protein Electrophoresis

Protein electrophoresis was performed on flat gels in a vertical chamber (E-C Apparatus Co., Philadelphia). Samples containing 100 μg protein, dissolved in 0.01 M phosphate buffer, pH 7.2, containing 5% sodium dodecylsulfate and 0.65 M mercaptoethanol, were applied to a 10% polyacrylamide gel (7 V/cm for 7 h). Tris-HCl buffer, 0.1 M, pH 8.0, containing 0.1% sodium dodecylsulfate served as electrode buffer and phenol red as indicator dye. Protein bands were stained with coomassie brilliant blue and absorbance tracings were recorded at 578 nm.

Electronmicrographs

All mitochondrial and submitochondrial fractions were examined by electron microscopy, using a Zeiss EM-9. The samples were fixed with chromate-osmic acid and contrasted with lead citrate.

Labeling Experiments

Amino Acid Incorporation in vitro. Isolated mitochondria were incubated with L-[^{14}C]leucine (312 mCi/mmol), L-[^{14}C]isoleucine (312 mCi/mmol) and L-[^{14}C]-phenylalanine (475 mCi/mmol) (Radiochemical Centre, Amersham), 0.25 μCi/ml each. Incubation was carried out under conditions described before [25].

Amino Acid Incorporation in vivo. Hemoglobin-free perfusion of rat livers was performed in a closed system with a modified Krebs-Henseleit bicarbonate buffer (150—200 ml), essentially as described by Sies and Brauser [26]. 5 min after addition of cycloheximide to the perfusion medium (500 μg/ml), 1 mCi of L-[^{3}H]leucine (150 mCi/mmol) was injected into the portal vein. Perfusion with the label was continued for 25 min. The perfusion medium was then made 1 mM with unlabeled leucine. Following 15 min of perfusion, the liver was cooled to 0 °C and mitochondria were prepared as described above. In control experiments no cycloheximide was added and 40 μCi L-[^{3}H]leucine was applied.

Measurement of Radioactivity

Aliquots of the different fractions containing 0.2—0.3 mg of insoluble protein were applied to wet Whatman glass-fibre filters and dried. The filters were then passed through 5% hot trichloroacetic acid (3 times), ethanol—ether, (3:1, by vol.), and ether. After drying the filters, radioactivity was determined in a Packard Tricarb scintillation counter in 15 ml toluene with PPO and POPOP.

RESULTS

Rat liver mitochondria were subfractionated by a combined technique of swelling, shrinking and

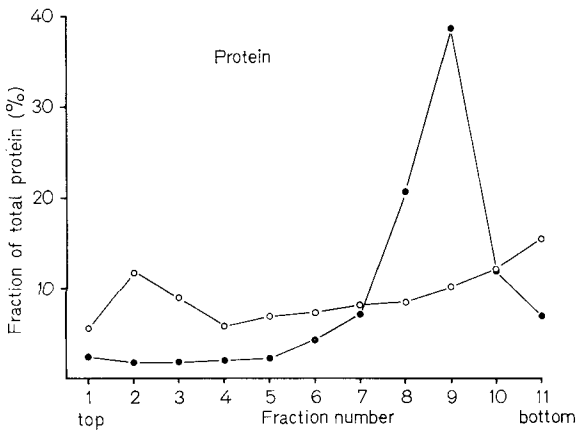


Fig. 1. *Distribution of insoluble mitochondrial protein in a linear sucrose-density gradient (1.13—1.25 g/ml).* Mitochondrial preparations were applied to the gradient before (●) and after (○) swelling, shrinking and sonication. Values are expressed as a percentage of total protein recovered from gradient

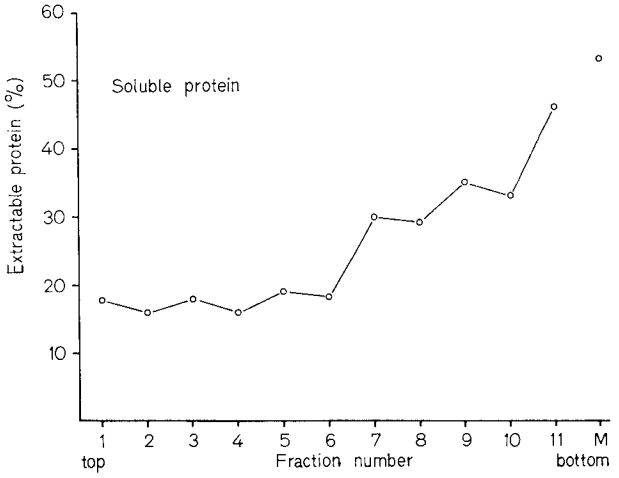


Fig. 2. *Proportion of soluble protein extractable by extensive sonication from different submitochondrial fractions, obtained by swelling, shrinking, sonication and subsequent sucrose-density-gradient centrifugation.* Untreated mitochondria (M) are included for comparison

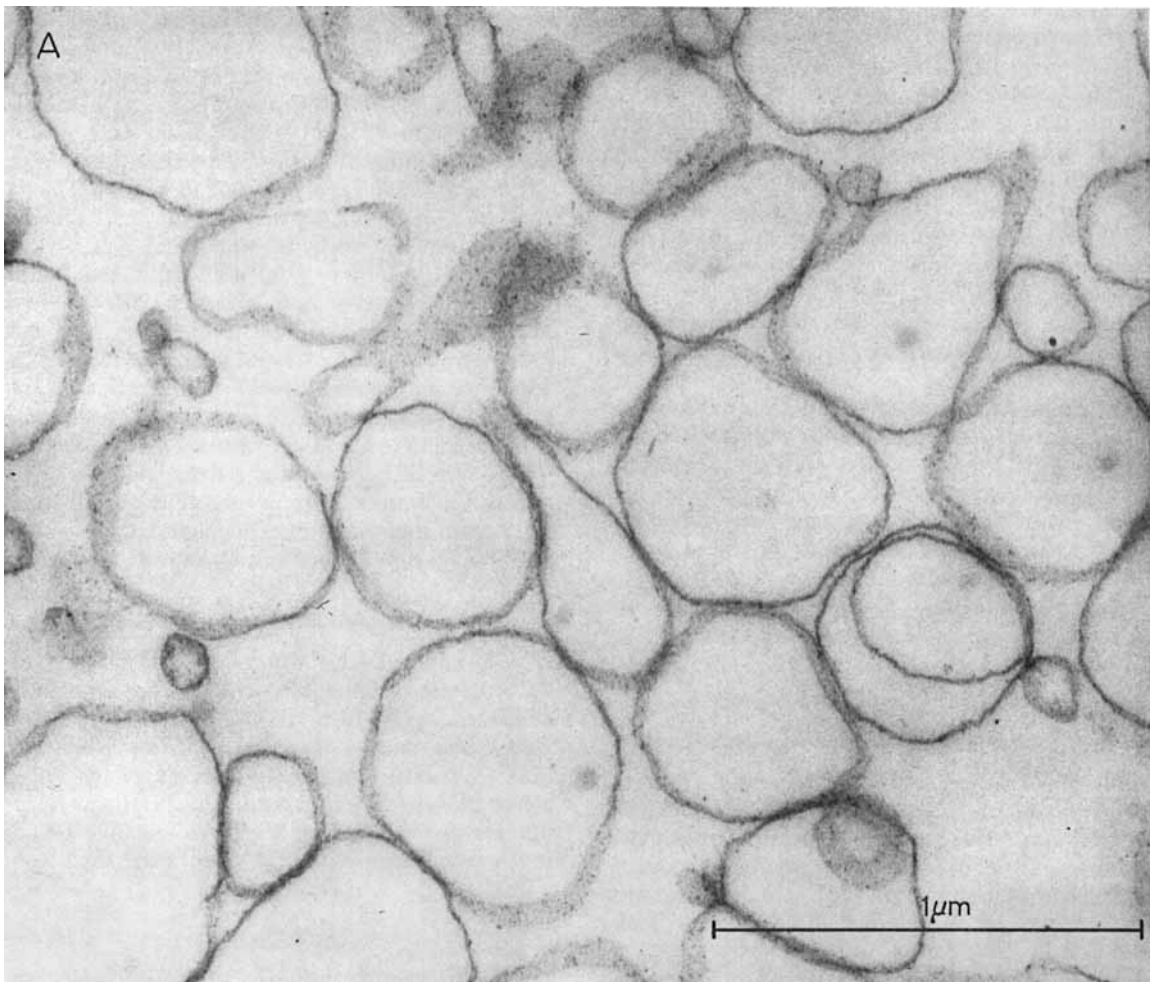


Fig. 3. *Electronmicrographs of different gradient fractions.* (A) Fraction 1; (B) fraction 3; (C) fraction 6; (D) fraction 11

sonication, followed by sucrose-density-gradient centrifugation.

The distribution of protein on the gradient before and after fractionation is shown in Fig. 1. Untreated mitochondria display one peak (fraction 9), whereas protein is largely dispersed throughout the gradient following fragmentation. Different amounts of soluble protein can be extracted from the different fractions by extensive sonication in phosphate buffer (Fig. 2). In the first six fractions the percentage extractable protein is low, as compared to whole mitochondria, whereas in fraction 11 it approximates the value obtained with untreated mitochondria.

Electronmicroscopic Appearance of Submitochondrial Fractions

Electronmicrographs of fractions 1, 3, 6, 11 are presented in Fig. 3. All fractions consist of single membrane vesicles. In fraction 1, the vesicles are entirely empty, while in fraction 3 some membranous material can be seen as inclusions. The vesicles in fraction 6 include a considerable amount of cristae-like structures, and fraction 11 contains an abundance of cristae profiles inside the vesicle membranes. By extensive sonication in phosphate buffer the particles of all fractions can be converted into tiny single membrane vesicles of about 0.05 to 0.1 μm diameter.

Enzyme Activities and Cytochrome Contents

In one series of experiments enzyme determinations were carried out with gradient fractions washed with phosphate buffer. In a second series gradient fractions were first sonicated and recentrifuged to give homogeneous particle preparations. This was done to exclude the possibility of variations in enzyme levels, due to variant degrees of membrane damage or due to different accessibilities for substrates. This treatment had no effect on the charac-

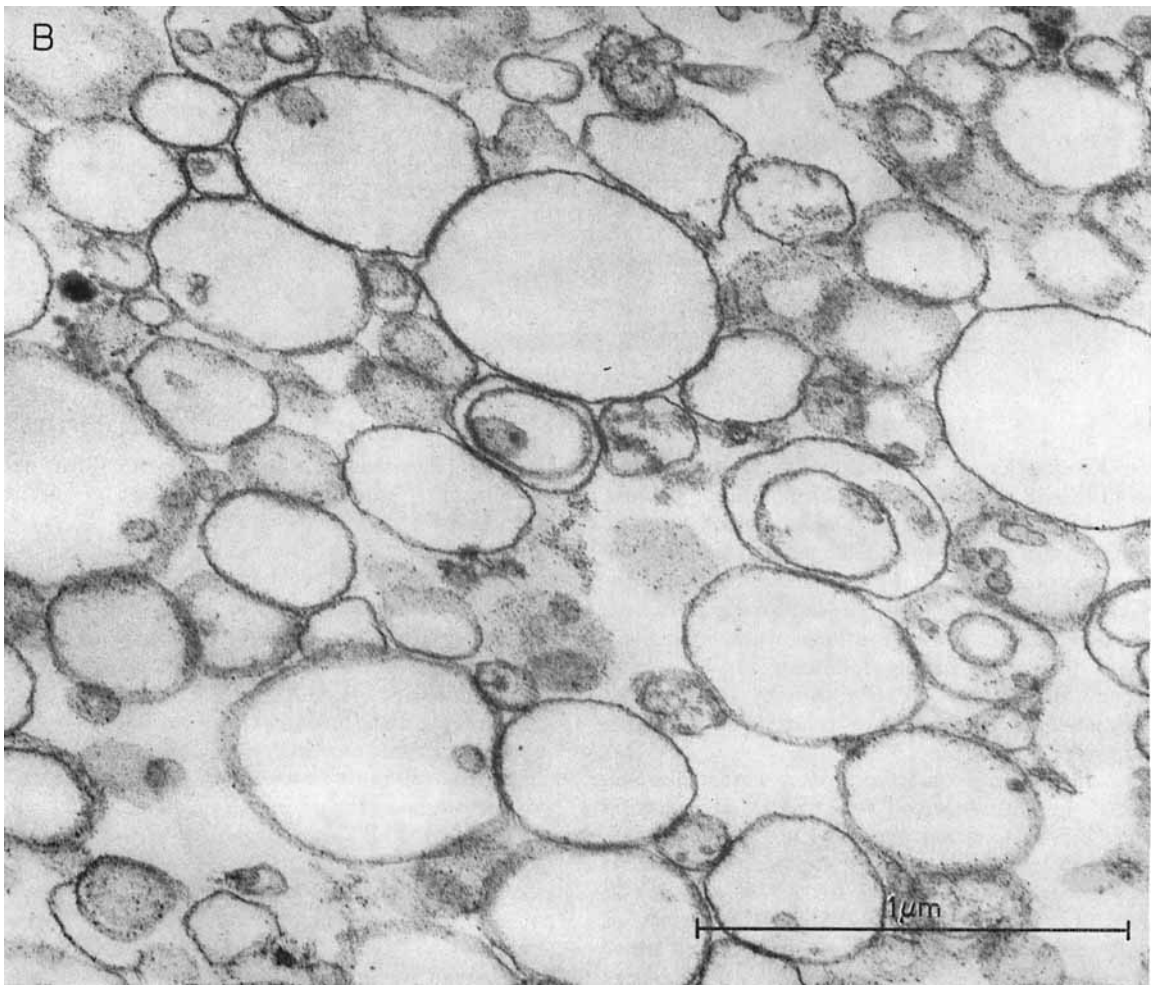


Fig. 3 B

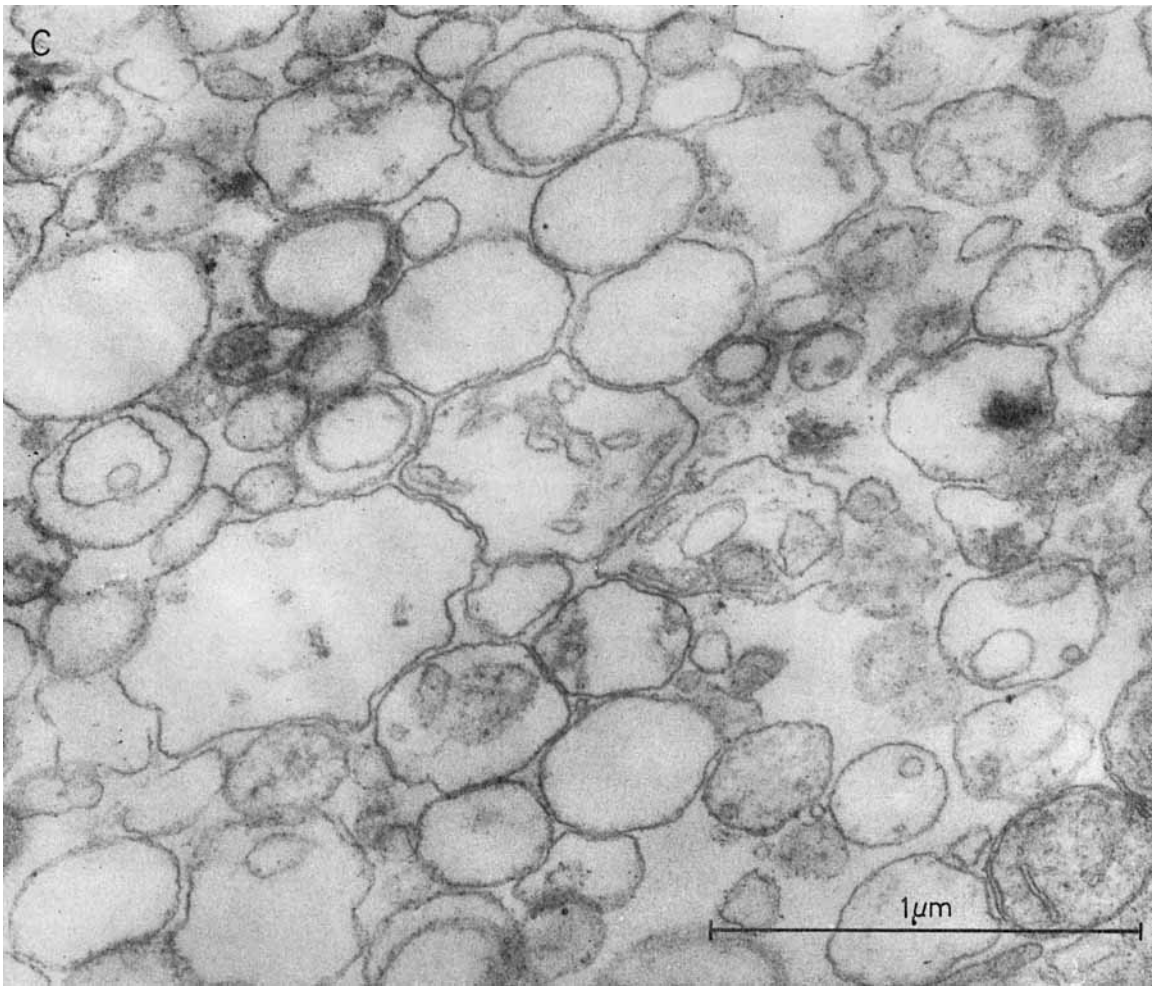


Fig. 3C

teristic distribution of the various enzyme activities in the gradient, when corrected for soluble protein contents. Also, no essential changes of specific enzyme activities were produced, except in the case of 3-hydroxybutyrate dehydrogenase, oligomycin-sensitive ATPase and cytochrome oxidase, where specific activities in all fractions were increased. The data presented in the following are based on the second procedure. All specific enzyme activities and cytochrome contents are related to insoluble membrane protein.

The distribution patterns of various enzyme activities are summarized in Fig. 4. Monoamine oxidase (Fig. 4A), a marker enzyme for the outer membrane [27, 28], is concentrated in fraction 1, declines abruptly and reaches its lowest activity in fraction 11. Conversely, the distribution curve of succinate-cytochrome *c* reductase (Fig. 4B), an inner membrane marker [9], rises from very low activities in the light fractions to maximal activity in fraction 11. Parallel to this enzyme runs the specific content in cytochrome aa_3 (Fig. 4C). Essentially the same behaviour is shown by the *b*-type cytochromes (Fig. 4C), which reach highest level in fraction 11. However, compared to cytochrome aa_3 a relatively high content was measured in the first fractions. This is due to cytochrome b_5, indicated by a shift of the α-band from 561 nm in fraction 11 to 556 nm in fraction 1—3.

Similar distributions are displayed by other inner membrane enzymes such as cytochrome oxidase (Fig. 4D), oligomycin-sensitive ATPase (Fig. 4E), proline oxidase (tested in the presence of cytochrome *c*) (Fig. 4F), and 3-hydroxybutyrate dehydrogenase (Fig. 4G).

A somewhat different behavior was observed with rotenone-sensitive NADH-cytochrome *c* reductase (Fig. 4H). Activity was nearly absent in the upper fractions of the gradient (1—4), but a drastic increase was observed in fractions 10 and 11.

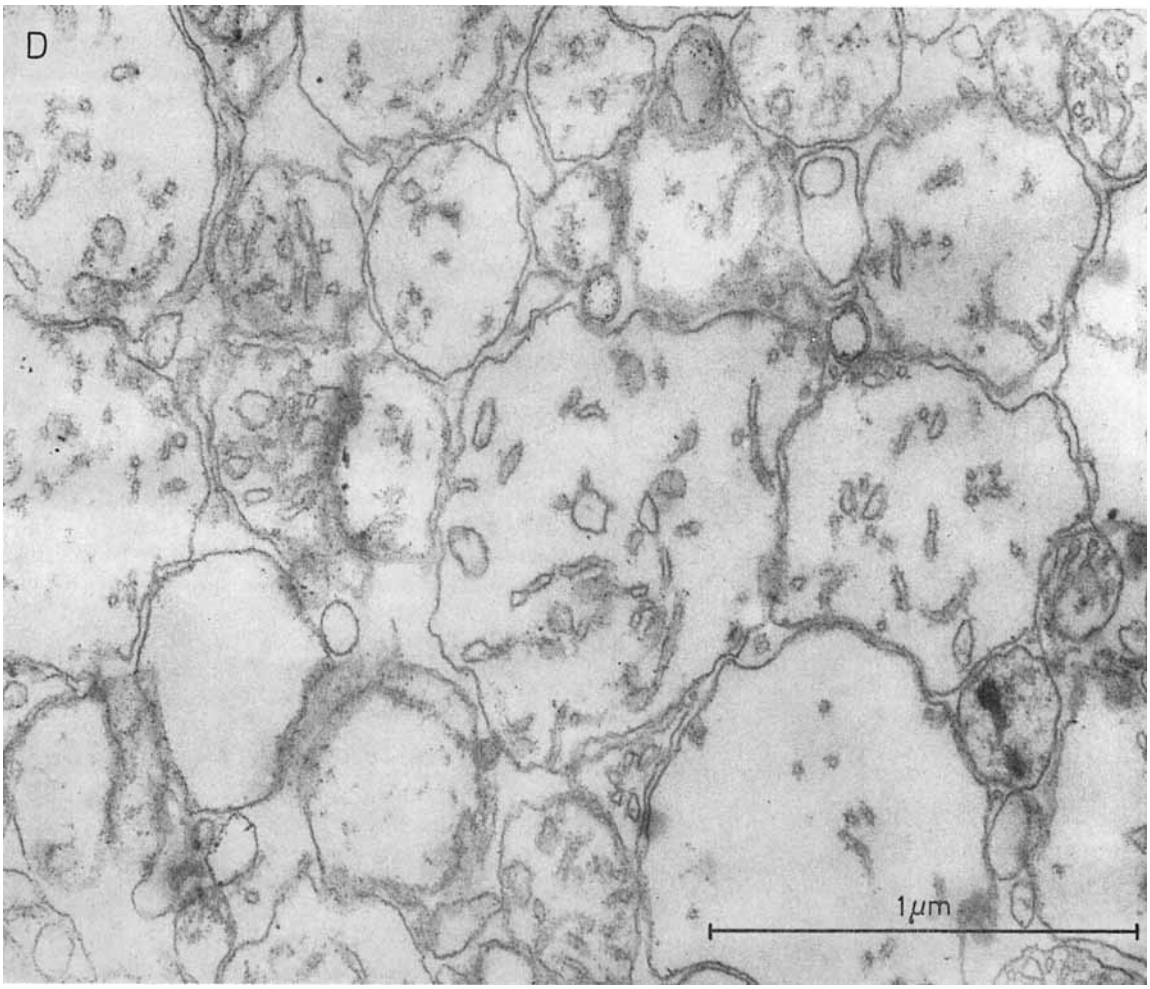


Fig. 3 D

Quite different distribution patterns were found for metal chelatase and glycerolphosphate dehydrogenase. The former enzyme reaches maximal activity in fraction 3 and stays at the same level in all following fractions. Fig. 4 I shows the data obtained by measuring the formation of cobalt · protoporphyrin. Essentially the same distribution of activity was found when the anaerobic formation of iron · protoporphyrin was measured. Glycerolphosphate dehydrogenase (Fig. 4 K) displays low activities in the top and bottom fractions, whereas peak activity is located in fraction 6. Glycerolphosphate dehydrogenase as well as succinate-cytochrome *c* reductase (see Fig. 4 A) were tested in the presence of various concentrations of phenazine methosulfate. Addition of this electron mediator resulted in an appreciable increase of specific activities of all fractions, however the distribution patterns were not changed with both enzymes.

Microsomal contamination was determined by measuring NADPH-cytochrome *c* reductase [9, 29] (Fig. 4 L). This contamination was highest in fraction 1, and decreased in subsequent fractions.

The distribution curve of rotenone-insensitive NADH-cytochrome *c* reductase (Fig. 4 M) declines more rapidly. This may be explained by the dual localisation of this enzyme in microsomes and outer mitochondrial membrane [9].

Finally the presence of soluble matrix proteins was checked using malate dehydrogenase (Fig. 4 N) as a marker. When gradient fractions were tested after density centrifugation and washing once with phosphate buffer, curve II was observed. When these fractions were sonicated, curve I was obtained. The enzyme is virtually absent in all fractions treated by sonication and recentrifugation (curve III, Fig. 4 N). This preparation corresponds to all others presented in Fig. 4. Obviously, the vesicles

Table 1. *Marker enzyme activities and cytochrome contents of mitochondria, characteristic submitochondrial fractions and microsomes*
Fractions 1, 3, 6, and 11 correspond to gradient fractions in Fig. 4 and fractions 1d, 3e, and 6d to those in Fig. 5

Fractions	NADPH-cytochrome *c* reductase	Cytochrome P-450	Cyto-chrome b_5	Monoamine oxidase	Succinate-cytochrome *c* reductase	Cyto-chrome aa_3	Metal chelatase	Glycerol-phosphate dehydro-genase	Metal chelatase/ succinate-cytochrome *c* reductase	Glycerolphosphate dehydrogenase/ succinate-cytochrome *c* reductase	Cytochrome aa_3/ succinate-cytochrome *c* reductase
	nmol × min^{-1} × mg^{-1}	nmol × mg^{-1}	nmol × mg^{-1}	nmol × min^{-1} × mg^{-1}	nmol × min^{-1} × mg^{-1}	nmol × mg^{-1}	nmol × min^{-1} × mg^{-1}	nmol × min^{-1} × mg^{-1}	‰	‰	‰
1	45.6	0.188	0.403	75.8	52	0.038	0.017	2.0			
1d	36.0			116.2	21			2.0			
3	30.1			12.1	162	0.130	0.652	11.1	4.0	69	0.80
3e	30.8			11.7	112		0.804	10.9	7.2	97	
6	11.1			10.7	490	0.362	0.696	60.5	1.4	123	0.74
6d	11.1			10.9	410		0.620	98.5	1.5	240	
11	3.33			5.0	988	0.685	0.716	22.1	0.7	22	0.70
Mitochondria	4.05			17.5	770	0.520	0.675	23.8	0.9	31	0.68
Microsomes	230.0	1.15	1.47								

obtained on the sucrose-density gradient contain different amounts of matrix proteins. Particles at the bottom of the tube contain large quantities of matrix proteins, whereas low amounts were found in the top fractions. Extensive sonication (curve I, Fig. 4N) liberates the enclosed matrix proteins, which then are accessible to NADH added to the assay system.

Fractions 1, 3 and 6 were subjected to further sucrose-density-gradient centrifugation. For each fraction such a gradient was employed that the density in the middle of the gradient equalled equilibrium density of the input fractions. This was calculated from the position in the first gradient (gradient A). The range of densities of these second gradients was roughly half of that of the first gradient. Fractions 1, 3, and 6 show peaks of protein distributions at sucrose densities of 1.12, 1.16, 1.19 g/ml, respectively. The values are in good agreement with those calculated from the first gradient. Fig. 5A—C show the protein distribution in these gradients as well as activities of enzymes specifically high in the particular input fractions.

After centrifugation of fraction 1 (Fig. 5A) highest specific activities of monoamine oxidase are

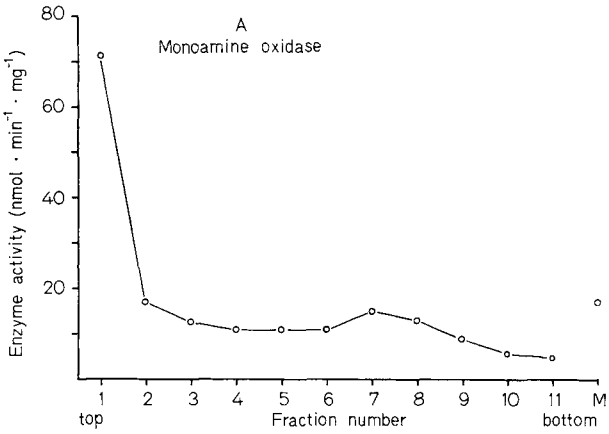
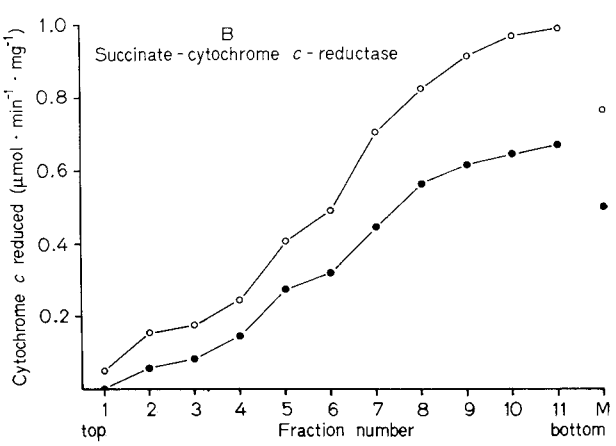
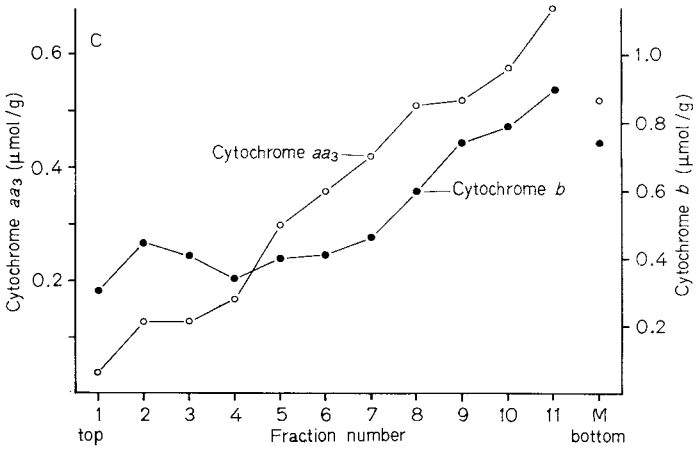
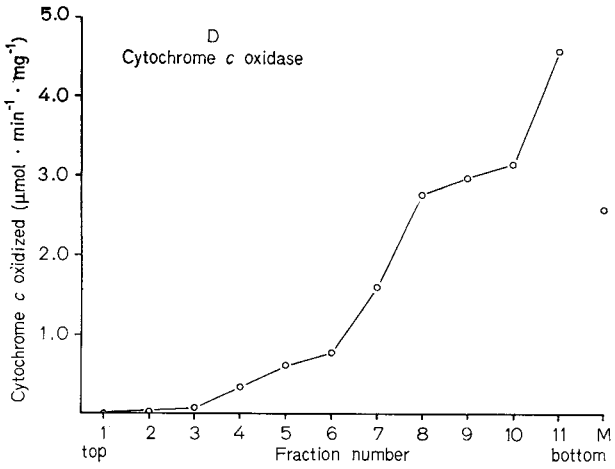
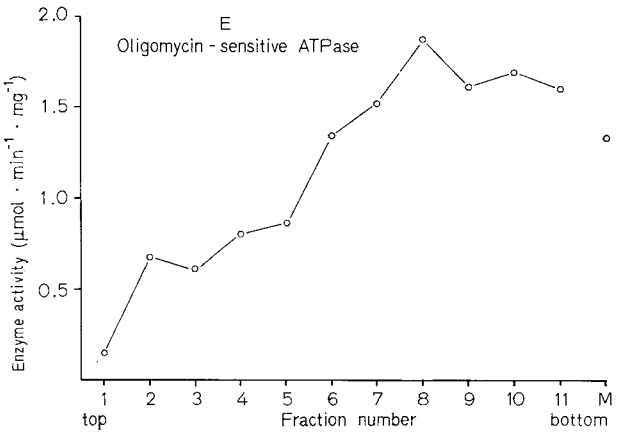


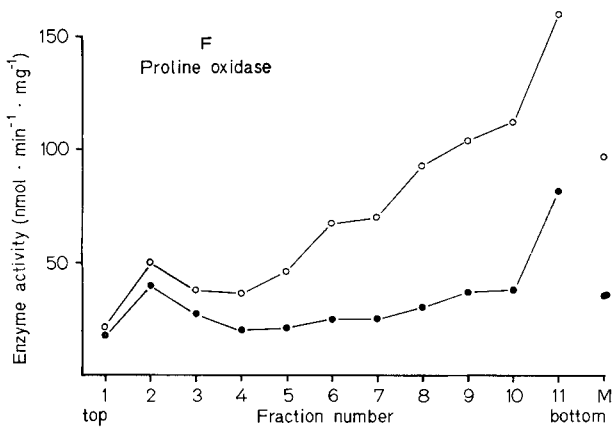
Fig. 4. *Distribution of enzyme activities and cytochrome contents in a linear sucrose-density gradient (1.13—1.25 g/ml), through which a mitochondrial preparation was centrifuged after swelling, shrinking and sonication.* Untreated mitochondria (M) are included for comparison. All values are related to insoluble protein. (A) Monoamine oxidase; (B) succinate-cytochrome *c* reductase in the presence (○) and absence (●) of phenazine methosulfate; (C) cytochrome aa_3 (○) and cytochrome *b* (●); (D) cytochrome oxidase; (E) oligomycin-sensitive ATPase; (F) proline oxidase, in the presence (○) and absence (●) of cytochrome *c*; (G) 3-hydroxybutyrate dehydrogenase; (H) rotenone-sensitive NADH-cytochrome *c* reductase; (I) metal chelatase; (K) glycerolphosphate dehydrogenase, in the presence (○) and absence (●) of phenazine methosulfate; (L) NADPH-cytochrome *c* reductase; (M) rotenone-insensitive NADH-cytochrome *c* reductase; (N) malate dehydrogenase, washed gradient fractions (●), *after sonication* (△), *after sonication* and recentrifugation (○)


B
Succinate - cytochrome c - reductase
Cytochrome c reduced (µmol · min⁻¹ · mg⁻¹)
1.0
0.8
0.6
0.4
0.2
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


C
Cytochrome aa3 (µmol/g)
0.6
0.4
0.2
Cytochrome aa3
Cytochrome b
Cytochrome b (µmol/g)
1.0
0.8
0.6
0.4
0.2
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


D
Cytochrome c oxidase
Cytochrome c oxidized (µmol · min⁻¹ · mg⁻¹)
5.0
4.0
3.0
2.0
1.0
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


E
Oligomycin - sensitive ATPase
Enzyme activity (µmol · min⁻¹ · mg⁻¹)
2.0
1.5
1.0
0.5
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


F
Proline oxidase
Enzyme activity (nmol · min⁻¹ · mg⁻¹)
150
100
50
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom

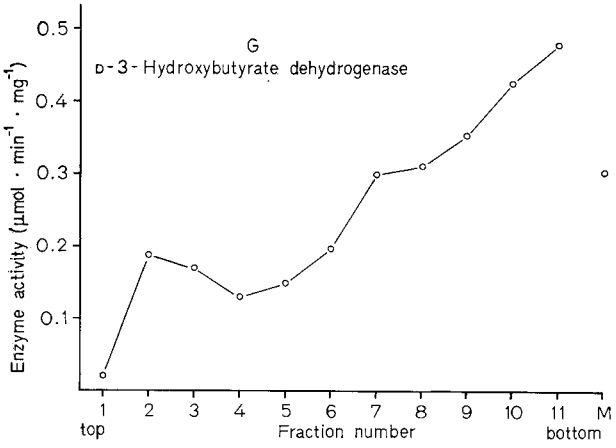
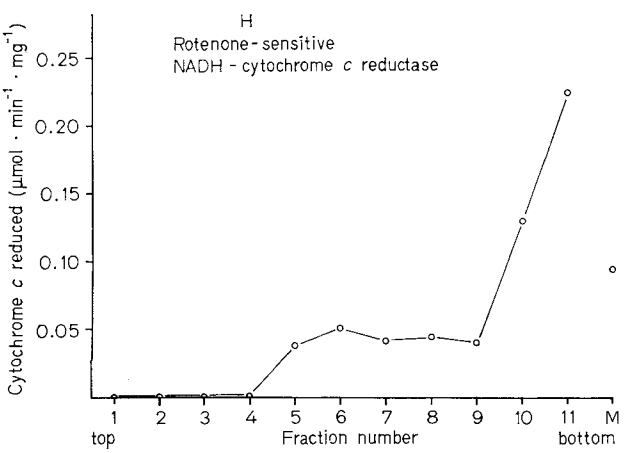
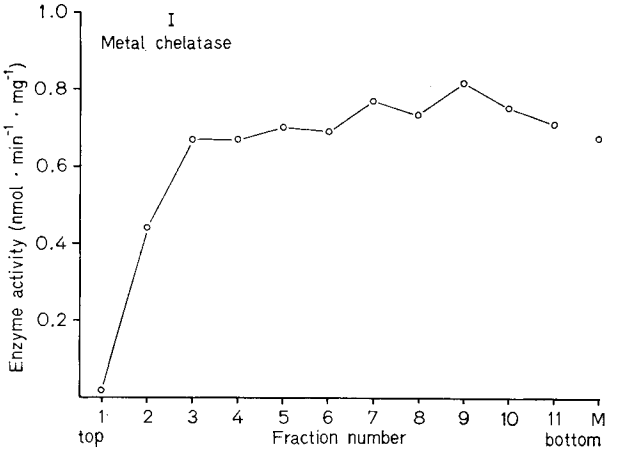
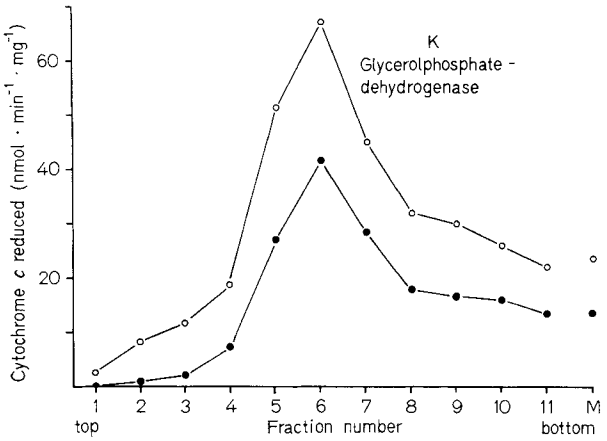
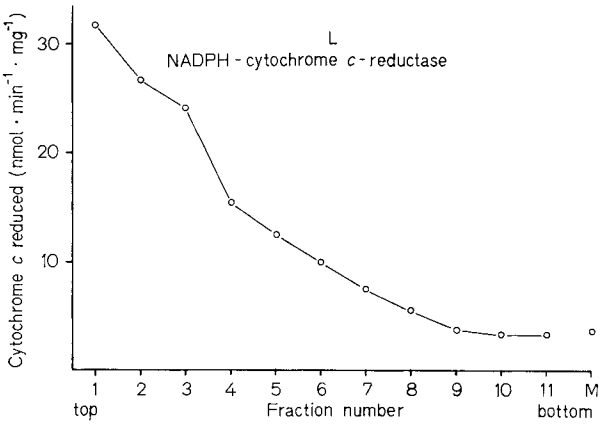

G
D-3-Hydroxybutyrate dehydrogenase
Enzyme activity (µmol · min⁻¹ · mg⁻¹)
0.5
0.4
0.3
0.2
0.1
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom

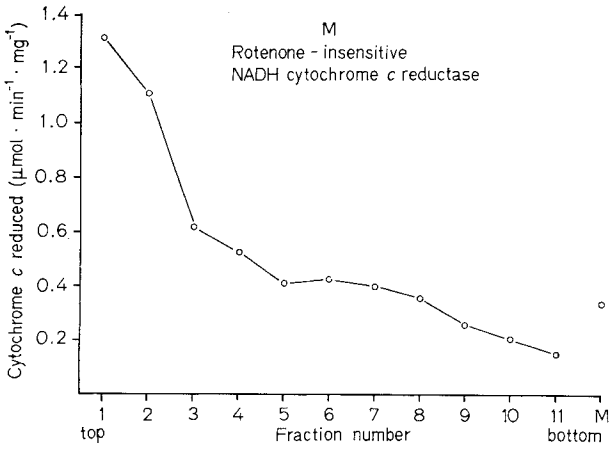
Fig. 4 B–G


H
Rotenone-sensitive
NADH - cytochrome c reductase
Cytochrome c reduced (µmol · min⁻¹ · mg⁻¹)
0.25
0.20
0.15
0.10
0.05
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


I
Metal chelatase
Enzyme activity (nmol · min⁻¹ · mg⁻¹)
1.0
0.8
0.6
0.4
0.2
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


K
Glycerolphosphate -
dehydrogenase
Cytochrome c reduced (nmol · min⁻¹ · mg⁻¹)
60
40
20
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


L
NADPH - cytochrome c - reductase
Cytochrome c reduced (nmol · min⁻¹ · mg⁻¹)
30
20
10
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom


M
Rotenone - insensitive
NADH cytochrome c reductase
Cytochrome c reduced (µmol · min⁻¹ · mg⁻¹)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom

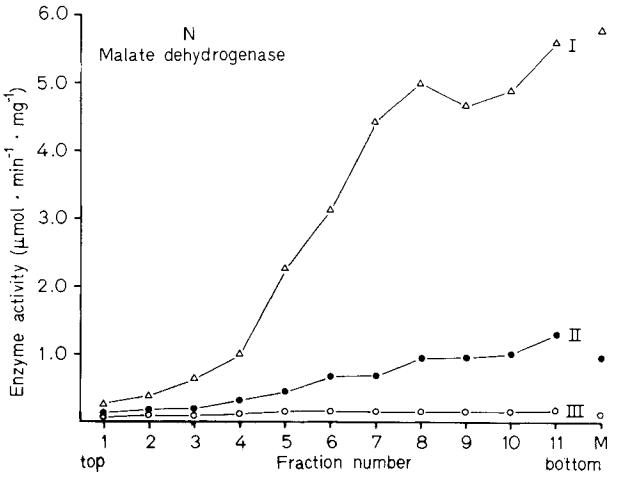
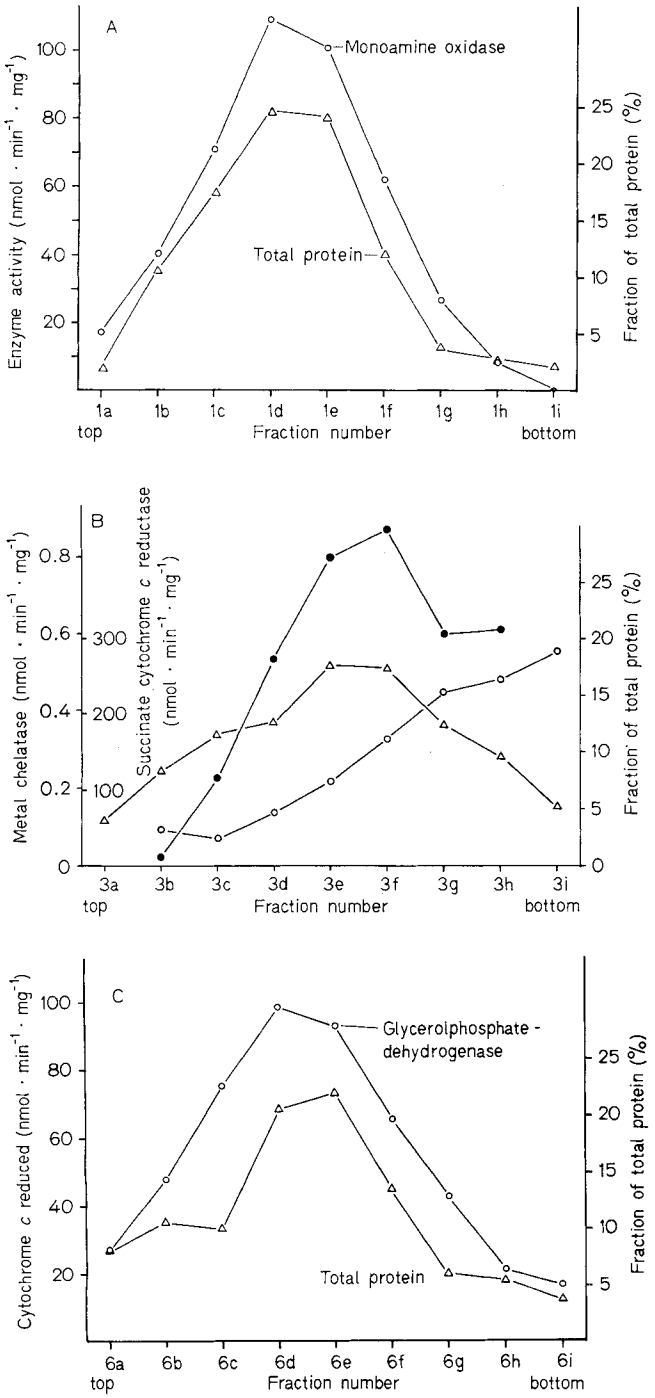

N
Malate dehydrogenase
Enzyme activity (µmol · min⁻¹ · mg⁻¹)
6.0
5.0
4.0
3.0
2.0
1.0
I
II
III
1 2 3 4 5 6 7 8 9 10 11 M
top
Fraction number
bottom

Fig. 4 H—N

found in fractions 1d and 1e. This exceeds the initial activity by about 50%. In the gradient to which fraction 3 was applied (Fig. 5B), metal chelatase was tested as a specific enzyme. It exhibits a peak at fractions 3e and 3f. The absolute specific activity of succinate-cytochrome *c* reductase in the input fraction is only 15% of that in fraction 11.



In contrast to metal chelatase this "contaminant" succinate-cytochrome *c* reductase activity (Fig. 5B) increases constantly in the lower fractions of the gradient. Fig. 5C shows the second gradient centrifugation of fraction 6, in which glycerolphosphate dehydrogenase shows a characteristically high level. This marker enzyme exhibits a maximum at fractions 6d and 6e. The bottom fractions of the gradient contain very low activities of glycerolphosphate dehydrogenase, whereas succinate-cytochrome *c* reductase is again highest there.

Table 1 summarizes enzymatic data on mitochondria, characteristic submitochondrial fractions and microsomes. The contamination of the individual fractions by microsomes can be calculated from the specific activities of NADPH-cytochrome *c* reductase. Whereas the contamination in whole mitochondria is as low as 2%, microsomes are enriched to 16% in outer mitochondrial membrane fractions (fractions 1 and 1d). Similar values for contamination of the outer membrane were observed from measurements of cytochrome P-450. Estimation of cytochrome P-450 levels in whole mitochondrial membranes and in inner membrane fractions was not possible, since spectral changes due to the carbon monoxide compound of cytochrome aa_3 (see [30]) interfere with those due to carbon monoxide binding to cytochrome P-450.

A dual localisation for cytochrome b_5 has been postulated in microsomes and outer membrane [31,32,9]. When fraction 1 is corrected for microsomal contamination on the basis of the values obtained for cytochrome P-450 and NADPH-cytochrome *c* reductase, a very low level (0.14 nmol/mg protein) for cytochrome b_5 in the outer membrane is calculated. This value is one half of that which is contributed to the outer membrane preparation by microsomal contamination. Certainly, cytochrome b_5 content of outer membrane, when extracted by sonication, is rather low. This is in agreement with observations of Sottocasa *et al.* [33] and Davis and Kreil [34], who described the removal of cyto-

Fig. 5. *Distribution of protein and enzyme activities in various sucrose-density gradients.* All values are related to insoluble protein. (A) Linear density gradient from 1.098 to 1.161 g/ml; input: fraction 1 (specific activity of monoamine oxidase was 71.1 nmol/min per mg protein); distribution of protein (△) and monoamine oxidase (○). (B) Linear density gradient from 1.132 to 1.182 g/ml; input: fraction 3 [specific activities of metal chelatase and succinate-cytochrome *c* reductase (+ phenazine methosulfate) were 0.652 and 162 nmol/min per mg protein, respectively]; distribution of protein (△), metal chelatase (●) and succinate-cytochrome *c* reductase (○). (C) Linear density gradient from 1.172 to 1.225 g/ml; input: fraction 6 (specific activity of glycerolphosphate dehydrogenase was 60.5 nmol/min per mg protein); distribution of protein (△) and glycerolphosphate dehydrogenase (+ phenazine methosulfate) (○)

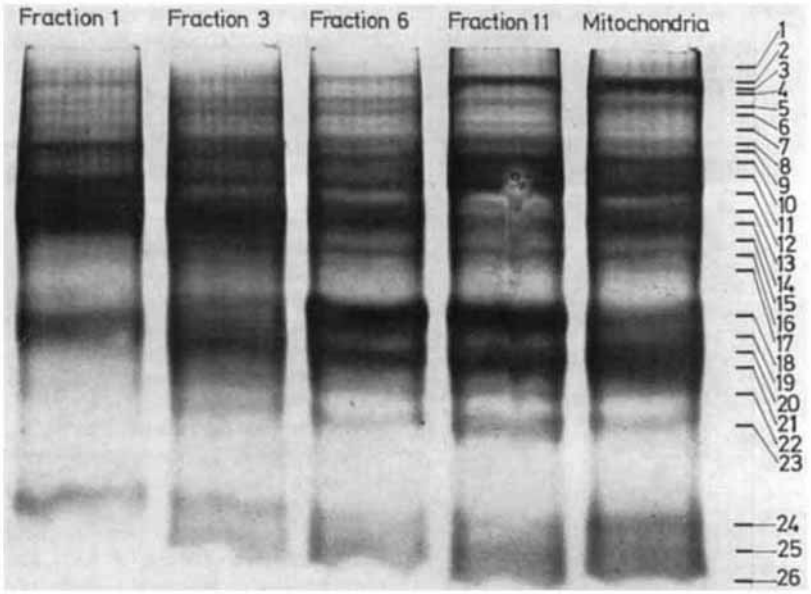


Fig. 6. *Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate of membrane proteins from mitochondria and submitochondrial fractions.* All samples were allowed to migrate on one gel. Staining was performed with coomassie brilliant blue

chrome b_5 from outer membrane by the swelling procedure or sonication.

The distribution of monoamine oxidase in the gradient shows a contamination of inner membrane fractions by outer membrane of between 4 and 10%. The high purity of outer membrane in fraction 1d allows an estimate of the portion of outer membrane protein of whole mitochondrial membranes. A value of 15% related to insoluble membrane protein is calculated.

Table 1 also lists the activities of the inner membrane markers metal chelatase, glycerolphosphate dehydrogenase, cytochrome aa_3, succinate-cytochrome *c* reductase and the ratios of the first three enzyme activities to the last one. The quotient metal chelatase/succinate-cytochrome *c* reductase shows a high value at fractions 3 and 3e and a low one in 11. The ratio glycerolphosphate dehydrogenase/succinate-cytochrome *c* reductase is highest in fractions 6 and 6d. It is lower in fractions 3 and 3d and comparatively very low in fraction 11 as well as in whole mitochondrial membranes. In contrast the ratio cytochrome aa_3/succinate-cytochrome *c* reductase is constant in all fractions of the gradients and is the same as in whole mitochondrial membranes. The enzyme ratios for fractions 1 and 1d were not calculated since the activities of the inner membrane enzymes are very low and therefore the ratios are subject to large errors.

Electrophoretic Protein Patterns

The fractions from the gradient as well as whole mitochondria and microsomes were subjected to sodium dodecylsulfate gel electrophoresis after extraction of soluble proteins. Fig. 6 shows a gel slab to which fractions 1, 3, 6, 11 and whole mitochondrial membranes were applied. All these fractions exhibit characteristic and highly reproducible band patterns. 26 different bands could be discriminated. In fraction 1 (outer membrane), bands 9, 12, 13/14 and 19 are predominant. The inner membrane fractions (3, 6, 11) show characteristic differences from the outer membrane fraction. They differ from each other in qualitative and quantitative terms. Whereas bands 13/14 are prevalent in fraction 3, they are low in fraction 6 and present in minute amounts in fraction 11. In contrast, band 18 is very low in fraction 3, but high in fraction 6 and fraction 11. Also band 21 is low in fraction 3 and increases from fraction 6 to fraction 11. Characteristic differences can also be observed with several other minor bands. The protein band pattern from whole mitochondrial membranes is clearly distinct from all submitochondrial membrane fractions. Information on detailed quantitative distribution of the bands is given in Fig. 7. It shows absorbance tracings of the stained gel. Besides the fractions presented in Fig. 6, a tracing of microsomal membrane proteins, run on the same gel is demonstrated. The microsomal pattern is clearly different

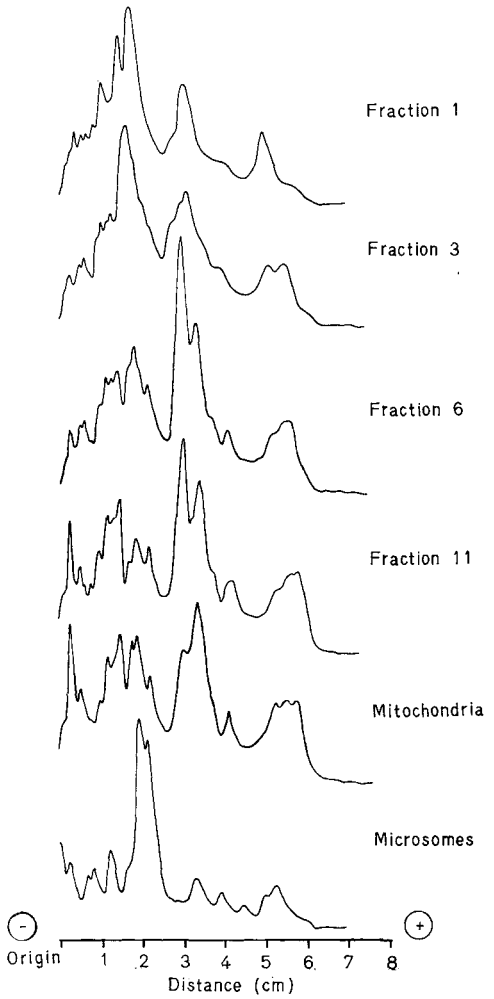


Fig. 7. *Absorbance tracings of electrophoretic patterns from membrane proteins of mitochondria, submitochondrial fractions and microsomes.* Absorbance of gels stained with coomassie brilliant blue was measured at 578 nm

from those of mitochondrial outer and inner membranes.

Phospholipid Content

Phospholipid content of the different submitochondrial fractions expressed as lipid phosphorus related to insoluble membrane protein is shown in Fig. 8. It is about twice as high in the outer membrane fraction compared to whole mitochondrial membranes and decreased with increasing fraction numbers. When the phospholipid content of the gradient fractions was determined in preparations from which soluble proteins were not extracted, this decrease was fairly linear.

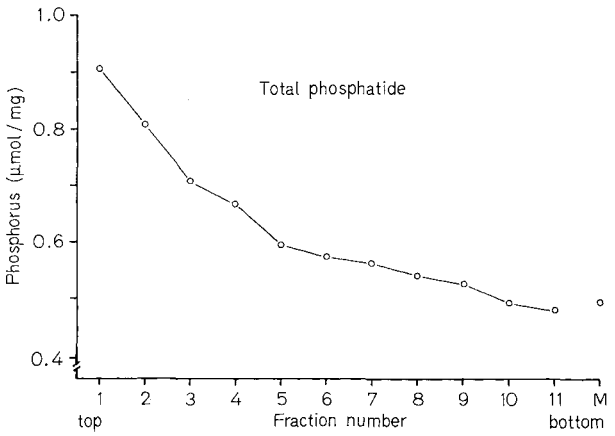


Fig. 8. *Phospholipid content of mitochondria (M) and submitochondrial fractions, obtained by swelling, shrinking, sonication and subsequent density-gradient centrifugation.* Values are related to insoluble protein

Amino Acid Incorporation into Membrane Fractions

Two different approaches were employed in attempts to determine the site of incorporation of labelled amino acids by the mitochondrial protein synthesizing system.

Incorporation of Amino Acids in vitro

Isolated mitochondria were incubated with radioactive leucine, isoleucine and phenylalanine under conditions for optimal amino acid incorporation [25], and subsequently subjected to the subfractionation procedure. The distribution of radioactivity incorporated into insoluble protein is presented in Fig. 9. Incorporation is low into the outer membrane, is also low into heavy inner membrane fractions. A distinct maximum of incorporation is found in fraction 6. A marked coincidence of the distribution of radioactivity and of glycerolphosphate dehydrogenase activity in the gradient is observed.

Incorporation of Amino Acids in vivo

To study the incorporation of amino acids into mitochondrial subfractions under conditions *in vivo*, livers were perfused with radioactive leucine in presence and absence of cycloheximide, a specific inhibitor of cytoplasmic protein synthesis [35—39]. As a control, livers were perfused without addition of the inhibitor. A 4-fold higher labeling of the outer membrane fraction than of the inner membrane fractions was observed (Fig. 10 A). This confirms earlier findings that the outer membrane has a higher turnover [40]. In other experiments the liver was perfused for 5 min with cycloheximide prior to

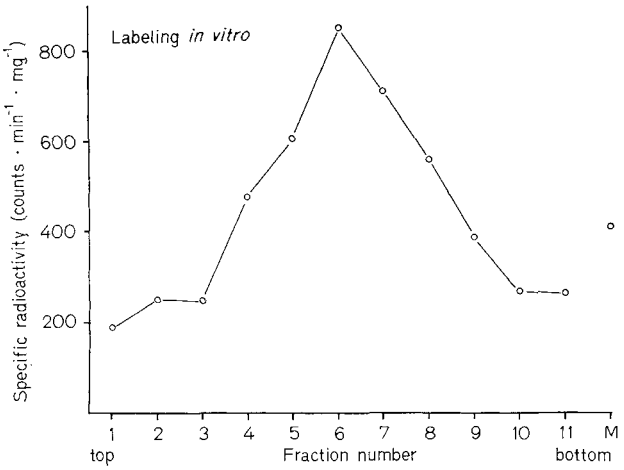


Fig. 9. *Specific radioactivities of mitochondrial membranes (M) and submitochondrial membrane fractions after incorporation of labeled amino acids into isolated mitochondria.* Fractions were prepared by swelling, shrinking, sonication and density-gradient centrifugation

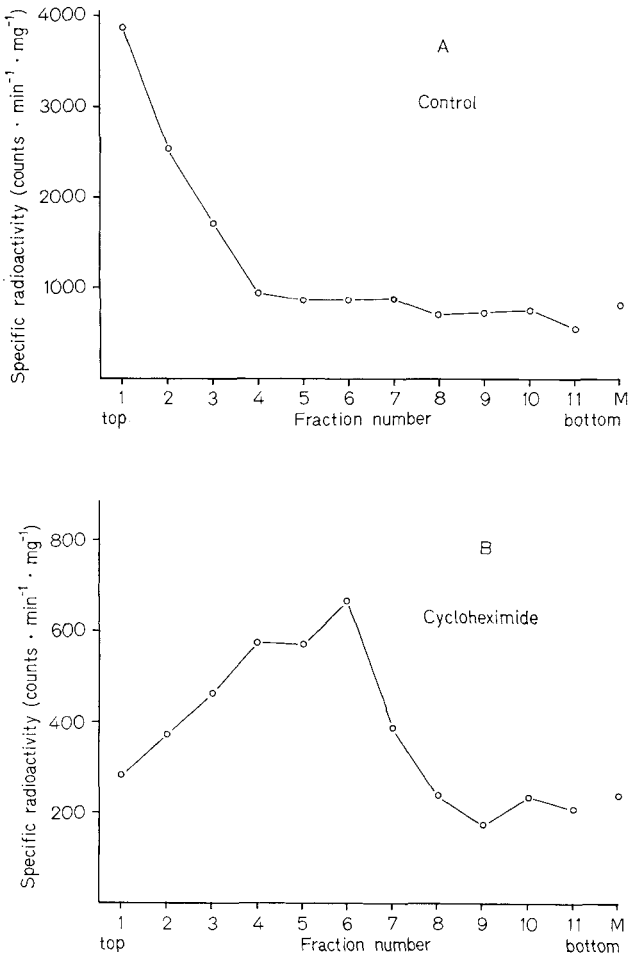


Fig. 10. *Incorporation* in vivo *of labeled amino acids into mitochondrial membranes (M) and submitochondrial membrane fractions prepared by swelling, shrinking, sonication and density-gradient centrifugation.* Livers were perfused with labeled amino acids in the absence (A) and presence (B) of cycloheximide. Specific radioactivities are related to insoluble protein

administration of radioactive leucine (Fig. 10 B). Incorporation into all fractions is drastically lowered, taking into account the 25-fold higher amount of radioactivity applied. However, the extent of cycloheximide inhibition differs within the gradient fractions. It is exceptionally high in the top fractions, also high in the bottom fractions, but lowest in fraction 6. Thus the distribution pattern of radioactive amino acids in the different submitochondrial fractions was the same *in vitro* as *in vivo* in the presence of cycloheximide.

DISCUSSION

Treatment of rat liver mitochondria by swelling, shrinking and sonication, followed by sucrose-density centrifugation results in the production and separation of membrane fractions with clearly different enzyme activity patterns. These differences cannot be ascribed to methodical artifacts, such as different degrees of membrane damage or different accessibility for substrates. Enzyme determinations were carried out after prolonged sonication of the gradient fractions. This treatment excludes differences due to sidedness, size and permeability of particles. Further proof for a structural heterogeneity of these particles comes from differences in phospholipid content and in the behavior of proteins on gel electrophoresis.

To explain the origin of these particles the following points must be taken into consideration. It could be argued that the observed separation reflects the occurrence of different types of mitochondria in the liver. Mammalian liver is composed of five distinct types of cells [41]. However, parenchymal cells constitute some 90—95% of total hepatic cellular weight [42]. Reticuloendothelial cells represent only 5—10% and their cytoplasm contains few mitochondria [43]. Bile duct, connective tissue and blood vessel wall cells can be neglected in this context as minor components. On the basis of these data it seems clear that mitochondria isolated from rat liver are essentially derived from parenchymal cells.

Furthermore, the possibility of a population of parenchymal cells with heterogeneous mitochondria has to be considered. Such a heterogeneity has been claimed on the basis of histological, biochemical and histochemical observations.

Different size of mitochondria in the periphery and in the center of the liver lobule has been described [44]. Also, isolation of mitochondria with different size and density has been reported [45,46]. However, the differences in density are very small (1.193 to 1.198 g/ml) [45]. Experiments with linear density gradients also indicate that mean particle diameters of mitochondria range only from 0.89 to 0.96 μm, when calculated from the midpoints of the distribution of various enzyme activities [47]. Thus, it can be excluded that these minimal differences, which may be even due to artifacts in the processing of the particles, cause a measurable heterogeneity in the gradient used in our studies. Actually, mitochondrial preparations applied to our linear density gradient showed a rather uniform distribution; production of heterogeneous particles occurred only after the procedure of swelling, shrinking and sonication. Moreover, if one fraction of the protein band resulting from density-gradient centrifugation of whole mitochondria was subjected to the subfractionation procedure, the same separation into particles with different densities and enzyme contents occurred.

More recently Schuel *et al.* [48] and Pollak and and Munn [49] reported the separation of rat liver mitochondria into two morphologically different populations by zonal and density-gradient centrifugation, respectively. The two types of mitochondria differed largely in equilibrium densities. However in both experiments, these two populations were the "condensed" and "orthodox" mitochondria, known for a long time, plus swollen or damaged mitochondria in a third band. Biochemical investigations produced no evidence for heterogeneity in either instance [49].

Heterogeneity of mitochondria in the liver has also been claimed on the basis of histochemical evidence. An unequal distribution of certain mitochondrial enzymes within the liver lobule has been described by various authors (for review see [50]). The activities of cytochrome oxidase [51] and succinate dehydrogenase [52,53] are reported to be greater in the periphery than in the center of the lobule, whereas glycerolphosphate dehydrogenase was found to be uniformly distributed [54]. However, conclusions can only be drawn from quantitative measurements. Such measurements have been only done for succinate dehydrogenase, for which a 1.5- to 1.6-fold higher activity was found in the periphery than in the center of the lobule [55]. This could mean that total inner membrane of mitochondria has a uniform structure in pericentral as well as in periportal cells, but there is a denser packing of enzyme molecules in mitochondria of the periportal cells. Another explanation for the histochemical finding is that the inner membrane is not uniformly structured. In this case the packing of the enzyme molecules on the inner membranes would be the same in all regions. Increased activity in the periportal area then is due to a relative enrichment of mitochondrial membranes within these cells.

It is possible to distinguish between these two concepts by quantitative electronmicroscopic measurements [56,57]. The percentage of mitochondrial volume of total cytoplasmic volume is higher in the peripheral cells by a factor of 1.5 [56]. On the other hand, the percentage of cristae volume of mitochondrial volume, and the ratio of cristae area to mitochondrial volume are quite constant [56]. The increase in mitochondrial volume is brought about by an increase in the size of individual mitochondria.

These data demonstrate that mitochondria from different parts of the lobule are not heterogeneous with respect to succinate dehydrogenase content per inner membrane area, but rather with respect to volume. This may reflect different metabolic functions of mitochondria, located in different parts of the liver lobule.

Of considerable interest in this context are investigations on mitochondrial membranes performed during a period of rapid mitochondrial morphogenesis. Gear [58] reported that the specific activities of marker enzymes for the inner membrane, and the patterns of mitochondrial protein electrophoresis remained constant during liver regeneration.

Based on these observations we are led to argue that the membrane fractions obtained, differing in their composition, are derived from heterogeneous inner membranes. This means, the inner membrane of a single mitochondrion is non-uniform in such a way that subfractionation results in the separation of structurally different parts.

Further support for this conclusion comes from morphological observations. It has been stressed by Allmann *et al.* [4] that projecting subunits apparent in negatively stained mitochondria are not evenly distributed over the whole inner membrane system. They are present on the cristae but not on the inner peripheral membrane (that part of the inner membrane which is adjacent to the outer membrane). In agreement with this, Vazquez *et al.* [5] observed two types of inner membrane structures, one so-called tubuli, entirely covered by projecting subunits and the other referred to as lamellae, free of projecting subunits. Racker *et al.* [59] studied the action of phospholipase C on whole mitochondria. These authors observed the formation of "sonic-particle-like" structures in relatively well preserved mitochondrial vesicles. This means that the enzyme preferentially attacked the cristae membranes, whereas the inner peripheral membrane was not affected.

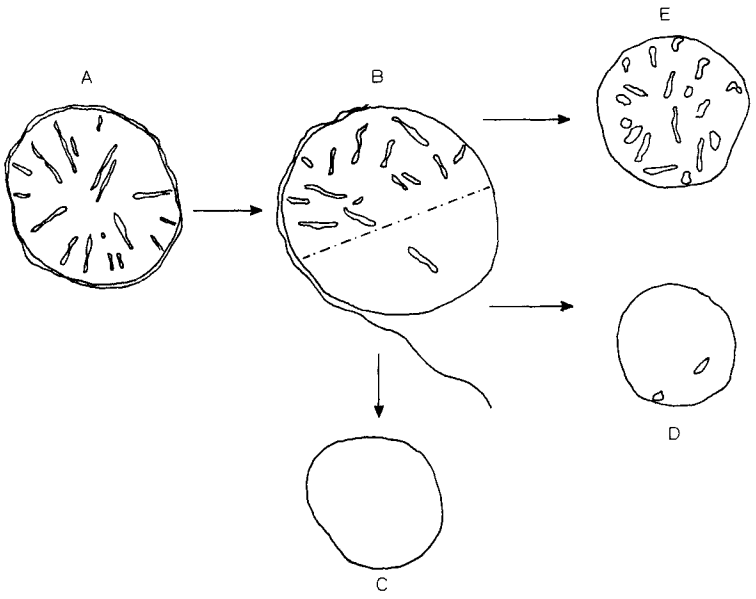


Fig. 11. *Suggested mechanism for the formation of different vesicular fractions from rat liver mitochondria by swelling, shrinking and mild sonication.* (A) Untreated mitochondria; (B) after swelling and shrinking; Mild sonication leads to fragments; (C) outer membrane; (D) inner peripheral membrane; (E) cristae membranes surrounded by inner peripheral membrane

Together with these observations, the morphological and biochemical data presented in this study led us conclude that the subfractions obtained represent different parts of inner mitochondrial membranes.

How can these fractions be correlated to the intact mitochondrion? There is abundant evidence that monoamine-oxidase-containing vesicles are derived from the outer membrane [27,28]. On the other hand, the progressive appearance of cristae profiles in the electronmicrographs of the heavy fractions of the gradient is paralleled by an increase of enzyme activities such as succinate-cytochrome *c* reductase, cytochrome oxidase and cytochrome aa_3 and *b* contents. The same holds for proline oxidase, oligomycin-sensitive ATPase, 3-hydroxybutyrate dehydrogenase and rotenone-sensitive NADH-cytochrome *c* reductase. This leads to the suggestion that these enzymes are localized predominantly in the cristae membranes.

The morphological appearance of the fractions between these two extremes is characterized by vesicles filled with different amounts of membranous material. It is suggested that the vesicles are derived from the inner peripheral membrane, retaining parts of the cristae membranes. The mechanism of their formation (Fig. 11) may be as follows: upon large amplitude swelling of mitochondria, cristae are disrupted to a large part from the inner peripheral membrane and are distributed randomly inside of rather enlarged vesicles formed by the inner peripheral membrane. Mild sonication then causes the disruption of these large vesicles into smaller ones. These will be filled with different amounts of cristae membranes. The enzymic composition of the empty vesicles (fraction 3, Fig. 3 B) should then reflect the enzymic composition of the inner peripheral membrane.

Two enzymes reveal distribution patterns quite different from those observed with the cristae markers. Metal chelatase is virtually absent in fraction 1 (outer membrane), but reaches maximal activity in fraction 3 and stays at the same level in all following fractions. On the basis of this behavior a dual localization for metal chelatase must be discussed, both in the inner peripheral and in the cristae membranes. A special problem rises with the distribution pattern of glycerolphosphate dehydrogenase. This enzyme is rather low in cristae-rich fractions. Furthermore, metal chelatase is already high in light fractions, while glycerolphosphate dehydrogenase has not reached its maximal activity. So it may be speculated that metal chelatase is distributed over the whole extent of the inner peripheral membrane, whereas glycerolphosphate dehydrogenase is concentrated at regions where cristae are connected to the inner peripheral membrane.

The phospholipid content of the outer membrane exhibits a rather high value on a protein basis (0.70 mg/mg protein), agreeing well with data reported by other authors [60—62]. Of special

interest is the large difference in phospholipid content between the inner peripheral membrane (0.55 mg/mg protein) and the cristae membranes (0.36 mg/mg protein).

The site of incorporation of radioactive amino acids by the mitochondrial system is the inner membrane. This has been shown with isolated rat liver mitochondria [25,63]. Data presented here demonstrate that also in perfused liver in the presence of cycloheximide incorporation occurs into the inner membrane. However, *in vitro* as well as *in vivo* the incorporation of radioactive amino acids does not follow cristae markers like succinate-cytochrome *c* reductase or cytochrome aa_3 and *b* levels. On the other hand, the distribution of the incorporated radioactivity coincides with that of glycerolphosphate dehydrogenase. These data suggest that newly synthesized polypeptide chains are inserted at specific regions of the mitochondrion, located on the inner peripheral membrane rather than on the cristae. We speculate that mitochondria contain "growth zones" at which the enzyme complexes of the inner membrane are assembled. This assembly would require a simultaneous supply of proteins and phospholipids synthesized inside as well as outside the inner membrane. It could be further speculated on the basis of our data that these regions of assembly are located at a position where the inner peripheral membrane merges into cristae membranes.

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