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Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the inner and outer membrane

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Rat liver mitochondria were isolated by a combination of differential and Percoll gradient centrifugation, resulting in a highly pure and intact preparation, as assessed by marker enzyme analysis, latency of cytochrome-*c* oxidase, respiratory control index and electron microscopy. Two different methods were compared for the separation of inner and outer membranes. In the swell-shrink-sonicate procedure glycerol was included resulting in the isolation of one outer membrane and two inner membrane fractions of high purity. Using digitonin a highly selective and gradual solubilization of the outer membrane could be accomplished. Analysis of the phospholipid composition of the intact mitochondria and all subfractions showed that the inner membrane was virtually devoid of phosphatidylinositol and -serine, while the outer membrane contained 23% of the total mitochondrial cardiolipin, which did not originate from inner membrane contamination and therefore is a true component of the outer membrane.

Introduction

Mitochondria have a limited capacity to synthesize membrane lipids and therefore depend largely on lipid import for membrane biogenesis [1]. In addition in some cases, membrane lipids are exported from the organel [1,2]. Flow of lipids between the inner and outer membrane is an important step in these processes. To study the biochemical and biophysical aspects of these transport processes in vitro requires preparation of well characterized, pure and functional mitochondria with an intact outer membrane as well as a rapid and quantitative method to separate inner and outer membrane.

Although many studies have been undertaken to achieve parts of these goals for rat liver mitochondria, to our opinion no satisfactory integral method resulting in quantitative data on lipid distribution between both membranes has yet been published. Therefore we undertook a systematic study, combining and adopting various published procedures, to isolate high quality rat liver mitochondria, subfractionate them via different methods and determine the lipid components and lipid content of both membranes.

A combination of differential and Percoll gradient centrifugation to isolate rat liver mitochondria in combination with controlled digitonin treatment for the removal of the outer membrane satisfied the above criteria. Based on marker enzyme activities, cardiolipin is found to be a true component of the outer membrane.

Materials and Methods

Materials

Digitonin was purchased from Merck (Darmstadt, F.R.G.) and was used without purification. Percoll was obtained from Pharmacia (Uppsala, Sweden). Essentially fatty acid free BSA was obtained from Sigma (St. Louis, U.S.A.). Other chemicals were of the best quality commercially available.

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Abbreviations: ADP, adenosine 5′-diphosphate; BSA, bovine serum albumine; EDTA, ethylenediaminetetraacetic acid; EGTA, (ethylenedioxy)-diethylenedinitrilotetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Methods

Isolation of rat liver mitochondria and microsomes. Male Wistar rats weighing approx. 300 g were starved overnight with water ad libitum. After killing the animal by cervical dislocation the liver was quickly removed and submerged in 250 mM mannitol, 0.5 mM EGTA, 5 mM Hepes, 0.1% (w/v) BSA (pH 7.4) (buffer A). Large cell debris and nuclei were pelleted by centrifuging twice for 5 min at $600 \times g_{max}$ in a Sorvall SS34 rotor. Mitochondria were pelleted by centrifuging the supernatant for 10 min at $10\,300 \times g_{max}$ in the same rotor. After suspending the pellet in 5 ml buffer A, the suspension was loaded on 4 tubes containing 20 ml 30% (v/v) Percoll in 225 mM mannitol, 1 mM EGTA, 25 mM Hepes, 0.1% (w/v) BSA (pH 7.4) and spun for 30 min at $95\,000 \times g_{max}$ in a Beckman 60Ti rotor. Mitochondria were collected from the lower part of the dense, brownish yellow mitochondrial band and were washed twice with buffer A by centrifuging 10 min at $6300 \times g_{max}$ in a SS34 rotor. Mitochondrial pellets were gently suspended in a small volume of buffer using an ice-cold, loosely fitting pestle. Microsomes were isolated from the supernatant of the crude mitochondrial pellet. After 10 min centrifuging at $27\,000 \times g_{max}$ in a SS34 rotor, microsomes were pelleted at $160\,000 \times g_{max}$ in a 60Ti rotor. The entire procedure was carried out at 0 to 4° C.

Subfractionation of rat liver mitochondria by the swell-shrink-sonicate procedure. Mitochondria were subfractionated by a modified combined procedure as described by Parsons et al. [3,4] and Sottocasa et al. [5]. A mitochondrial pellet obtained from two livers was suspended in 10 mM KH_2PO_4 (pH 7.4) to a total volume of 6 ml. After incubating 15 min with stirring, 6 ml 32% (w/v) sucrose, 30% (v/v) glycerol, 10 mM $MgCl_2$ in 10 mM KH_2PO_4 (pH 7.4) were added and the suspension was incubated another 15 min. The suspension was sonicated twice for 15 s with a 1-min interval using a Branson B-12 sonicator equiped with a 5 mm diameter tip operating at 60 to 70 watt. The mixture was centrifuged 10 min at $12\,000 \times g_{max}$ in a SS34 rotor, whereafter pellet and supernatant were separated. The pellet, after suspending in 8 ml buffer A, and the supernatant were loaded on separate discontinuous sucrose gradients composed of 2 ml each of 25.3, 37.7 and 51.3% (w/v) sucrose in 10 mM KH_2PO_4 (pH 7.4) and spun for 3 h at $210\,000 \times g_{max}$ in a Beckman SW 41Ti rotor. Three fractions were collected: one at the 25.3/37.7% interface of the gradient on which the supernatant was loaded, one in the 51.3% layer and one at the bottom of the gradient on which the pellet was loaded. The first fraction was washed with buffer A by centrifuging 1 h at $160\,000 \times g_{max}$ in a 60Ti rotor and the other two by centrifuging 10 min at $12\,000 \times g_{max}$ in a SS34 rotor. These fractions were called OM, IM and IMM, respectively, because OM is highly enriched for the outer membrane marker monoamine oxidase, IM for the inner membrane marker and IMM for both inner membrane and matrix markers. The entire procedure was performed at 0 to 4° C.

Subfractionation of rat liver mitochondria by digitonin. The procedure, described by Schnaitman and Greenawalt [6], was used with some essential modifications, the most important one being the lower mitochondrial protein concentration. Shortly, to a suspension of 20 mg mitochondrial protein per ml in 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.05% (w/v) BSA (pH 7.4) (buffer B) an equal volume of buffer B without BSA containing the desired amount of digitonin was added. After incubation for 15 min on ice the sample was diluted 10-times with buffer B and centrifuged for 10 min at $12\,000 \times g_{max}$ in a SS34 rotor. Pellet and supernatant were stored separately at −20° C for further analysis. Only at a digitonin/protein ratio of 0.3 (w/w) a fluffy layer was present, which was not removed from the pellet.

Enzyme assays. Succinate–cytochrome-*c* reductase activity was measured in the medium described in [7] for succinate dehydrogenase except that phenazine methosulfate was omitted and 2 μM rotenone and 50 μM cytochrome *c* were added.

Cytochrome-*c* oxidase activity was measured by following the oxidation of 50 μM reduced cytochrome *c* in a medium containing 130 mM KCl, 10 mM Hepes (pH 7.4), 0.5 mM EDTA, 2 μM rotenone, 2 μM carbonylcyanide *m*-chlorophenylhydrazone, in the absence or presence of 0.2% Lubrol PX. Complete oxidation of the cytochrome *c* was achieved by adding 1 mM $K_3Fe[CN]_6$. Latency of cytochrome-*c* oxidase was defined as [1 − (rate without Lubrol/rate with Lubrol)]. Ferrocytochrome *c* was obtained by reducing cytochrome *c* with ascorbate and by removing the reductant by gel filtration.

Monoamine oxidase [8], acid phosphatase [9], catalase [10] and 5′-nucleotidase [11] were measured according to published methods. Adenylate kinase was measured in the presence of 25 μg/ml oligomycin according to Ref. 12. Fumarase was measured as in Ref. 13 with or without 0.4% Lubrol PX. Glucose-6-phosphatase was measured as described in Ref. 14 with 14 mM histidine, 2 mM EDTA as buffer. Marker enzyme activities were almost quantitatively recovered during the course of isolations and subfractions.

Respiration was followed using a Yellow Springs Instrument Model 5331 oxygen probe in a medium of 130 mM KCl, 3 mM Hepes, 2 mM KH_2PO_4, 4 mM succinate, 2 μM rotenone (pH 7.4) at 25° C. Routinely, 1.5 mg mitochondrial protein was added to 3 ml medium. Respiratory states were as defined by Chance and Williams [15]. State III was induced by adding 0.16 or 0.33 mM ADP. The P/O ratio was determined according to Estabrook [16].

Lipid determinations. Lipids were extracted by the

method of Bligh and Dyer [17]. Samples, containing up to 1 μmol lipid phosphorus were extracted in a total volume of 6 ml. After phase separation, the chloroform phase was collected and the methanolic phase and the interphase were extracted twice with 2 ml of chloroform. The combined extracts were evaporated to dryness and the lipids were dissolved in chloroform. Phospholipid species were separated by one- or two-dimensional thin-layer chromatography [18] (for more details see Fig. 3). Lipid phosphorus was determined after destruction with 70% perchloric acid by the method of Fiske and SubbaRow [19]. Recovery of phospholipids from the thin-layer plates was always near 100%.

Electron microscopy. Freeze substitution and low temperature embedding in Lowicryl HM20 were performed according to Ref. 20. Thin sections were stained either with 2% aqueous $KMnO_4$ for 3 min or with saturated aqueous uranyl acetate for 5 min followed by lead acetate according to Millonig [21]; both staining methods resulted in comparable morphologies. Thin sections were viewed using a Philips 201 or 301 electron microscope operated at 60 kV.

Protein determination. Protein was determined by the BCA assay of Pierce Chemical Company (Rockford, U.S.A.) including 0.1% (w/v) sodium dodecylsulfate with BSA as standard. Recovery of protein was always near 100%.

Results

Isolation and characterization of rat liver mitochondria

The need to obtain intact and pure mitochondria imposes the use of density gradient centrifugation to remove contaminating organels and broken mitochondria. The gradient medium best suited is Percoll, because of its almost negligible osmolarity and metabolic inertness [22].

Table I compares both absolute and relative (with respect to the homogenate) specific activities of several marker enzymes in the final preparation of purified mitochondria. The mitochondrial markers are enriched 2.2–5.6-times, the extremes being the matrix marker fumarase and the inner membrane marker succinate–cytochrome-*c* reductase, respectively. This range of the relative specific activities could be due to the existence of mitochondrial subpopulations in the liver with different enzymatic characteristics [23] and/or the possible nonexclusive mitochondrial localization of certain marker enzymes, as was recently suggested for yeast fumarase [24]. The preparation is deprived in microsomes, lysosomes, plasma membrane and peroxisomes, as judged by the specific activities of the marker enzymes glucose-6-phosphatase, acid phosphatase, 5′-nucleotidase and catalase, respectively. Isolated microsomes show a (23 ± 9)-times higher specific activity of glucose-6-phosphatase when compared with the mitochondrial preparation ($n = 11$), indicating that on a protein basis the mitochondrial preparation contains some 4% microsomes. Based on the specific activities of 5′-nucleotidase [25], catalase [26] and lysosomal markers [27] for the isolated organels, our mitochondrial preparation contains approx. 1% (on protein basis) of plasma membrane, peroxisomes and lysosomes. The relative moderate decrease in the specific activity of the lysosomal marker acid phosphatase is as expected, because of the similar buoyant densities of lysosomes and mitochondria [28]. Of the total protein present in the homogenate (4.7 ± 1.7)% is recovered in the final preparation ($n = 20$), thus (12.2 ± 5.5) to (26 ± 12)% of the mitochondria are recovered, based on the total activities of fumarase and succinate–cytochrome-*c* reductase, respectively. Intactness of the outer membrane as determined by the latency of cytochrome-*c* oxidase was (89.4 ± 2.7)% ($n = 20$). Both the respiratory control in-

TABLE I

Enzymatic characterization of the isolated rat liver mitochondria

Mitochondria were isolated by the method as described in Materials and Methods. Data are presented as means ± S.D. and the number of experiments is given between parenthesis.

Enzyme	Location	Specific activity (nmol/min per mg protein)	Specific activity relative to the homogenate
Mitochondrial markers			
Monoamine oxidase	outer membrane	13.1 ± 4.7	2.6 ± 0.7 (28)
Adenylate kinase	intermembrane space	450	2.5 ± 0.4 (3)
Succinate–cytochrome-*c* reductase	inner membrane	220 ± 70	5.6 ± 1.4 (18)
Cytochrome-*c* oxidase	inner membrane	[a]	5.2 ± 1.1 (9)
Fumarase	matrix	600 ± 200	2.2 ± 0.3 (4)
Non-mitochondrial markers			
Glucose-6-phosphatase	microsomes	26 ± 14	0.18 ± 0.07 (18)
Acid phosphatase	lysosomes	$(90 \pm 40) \cdot 10^{-3}$	0.7 ± 0.1 (4)
5′-Nucleotidase	plasma membrane	26 ± 3	0.33 ± 0.12 (5)
Catalase	peroxisomes	$60 \cdot 10^3$	0.42 ± 0.12 (3)

[a] (Δlog absorbance)/min per mg protein = 66 ± 7.

TABLE II

Enzymatic characterization of the mitochondrial subfractions obtained by the swell-shrink-sonicate procedure

Fractions were isolated as described in Materials and Methods. OM stands for the isolated outer membrane fraction, IM for the inner membrane fraction and IMM for the inner membrane plus matrix fraction. Data are presented as means ± S.D. and the number of experiments is given between parenthesis.

Enzyme	Specific activity relative to the mitochondria		
	OM	IM	IMM
Monoamine oxidase	14 ±3 (21)	0.6 ±0.3 (19)	0.4 ±0.2 (23)
Succinate–cytochrome-*c* reductase	0.23 ± 0.18 (19)	4.5 ±1.6 (18)	2.2 ±1.1 (17)
Fumarase	0.01 ± 0.01 (3)	0.30 ±0.17 (3)	2.4 ±1.6 (3)
Adenylate kinase	0.25 ± 0.03 (2)	0 (1)	0.03 (1)
Glucose-6-phosphatase	3.3 ±1.5 (11)	0.24 ±0.07 (9)	0.08 ± 0.04 (9)
Acid phosphatase	8.3 ±0.5 (2)	0.44 ±0.04 (2)	0.21 ± 0.14 (2)
5′-Nucleotidase	9.5 ±1.0 (2)	0.075 ± 0.007 (2)	0.12 ± 0.09 (2)

dex of 5.4 ± 0.6 ($n = 29$), with an oxygen consumption of 120 ± 30 nmol O/mg protein per min in state III ($n = 9$), and the P/O ratio of 2.07 ± 0.14 ($n = 10$) are indicative of a high degree of coupling between respiration and phosphorylation [29].

Figs. 1A and 1B show electronmicrographs of thin sections of a mitochondrial suspension, after freeze substitution and low-temperature embedding. In Fig. 1A almost all profiles can be attributed to mitochondria, in agreement with the high purity of the preparation. Fig. 1B shows the typical ultrastructure of these mitochondria: the inner boundary membrane and the outer membrane are in very close approximation, judged from the pentalamellar appearance, which agrees with the morphology in tissue after freeze substitution [30]. Freeze-fracture electron microscopy confirmed the morphology of the mitochondrial preparation (data not shown).

Subfractionation of rat liver mitochondria via the swell-shrink-sonicate procedure

Osmotic and mechanical methods to disrupt mitochondria in order to separate the mitochondrial inner and outer membrane might benefit from the action of substances, which induce both a pronounced shrinkage of the matrix and a loss of contact between the inner and outer membrane. For this reason we included glycerol in the hypertonic shrink medium, since it has been shown that glycerol causes a large separation of both membranes and diminishes the contacts between them [31].

When the swell-shrink-sonicate procedure (see Methods) is applied three fractions are isolated, which contain approx. 30% of the protein applied to the gradients. The remainder is quantitatively recovered in the sucrose gradients, outside the bands from which the fractions were collected, and in the supernatant obtained during the washing of these fractions. The fraction OM, collected from the 25.3/37.7% (w/v) sucrose interface of the gradient on which the supernatant of the low-speed centrifugation step was loaded, has a 14-fold higher specific activity of the outer membrane marker monoamine oxidase than the parent mitochondria (Table II). The specific activities of the inner membrane marker succinate–cytochrome-*c* reductase and adenylate kinase, an inter membranous space enzyme, are decreased approx. 4-fold, while the specific activity of the matrix marker fumarase is negligible. These findings show that fraction OM is an outer membrane preparation, containing approx. 5% inner membrane, based on the specific activity of succinate–cytochrome-*c* reductase in OM and IM. Contact site fractions, which are enriched in both inner and outer membrane markers, as isolated from rat liver mitochondria [32], would be recovered in this fraction based on their density and could be responsible for the low contamination with inner membrane. Based on the total activity of monoamine oxidase (21 ± 8)% of the outer membrane is recovered in this fraction ($n = 21$). In Table II it is shown that the contaminations, present in the starting mitochondrial preparation, tend to copurify with the outer membrane. Based on the specific activities of glucose-6-phosphatase, lysosomal markers [27] and 5′-nucleotidase [25] this fraction contains 13, 8 and 10% on protein basis of microsomes, lysosomes and plasma membrane, respectively. Fig. 1C shows an electronmicrograph of the outer membrane fraction; small vesicles ranging from 50 to 350 nm in diameter are seen, sometimes oligolamellar.

The IMM and IM fractions are both enriched in the inner membrane marker succinate–cytochrome-*c* reductase and depleted in the outer membrane marker monoamine oxidase, indicating that these fractions are inner membrane fractions. The high relative specific activity of fumarase indicates that IMM is an inner membrane plus matrix fraction. Based on the specific activity of monoamine oxidase, IM and IMM contain 4 and 3% outer membrane (on protein basis). Contamination from nonmitochondrial origin is low as judged

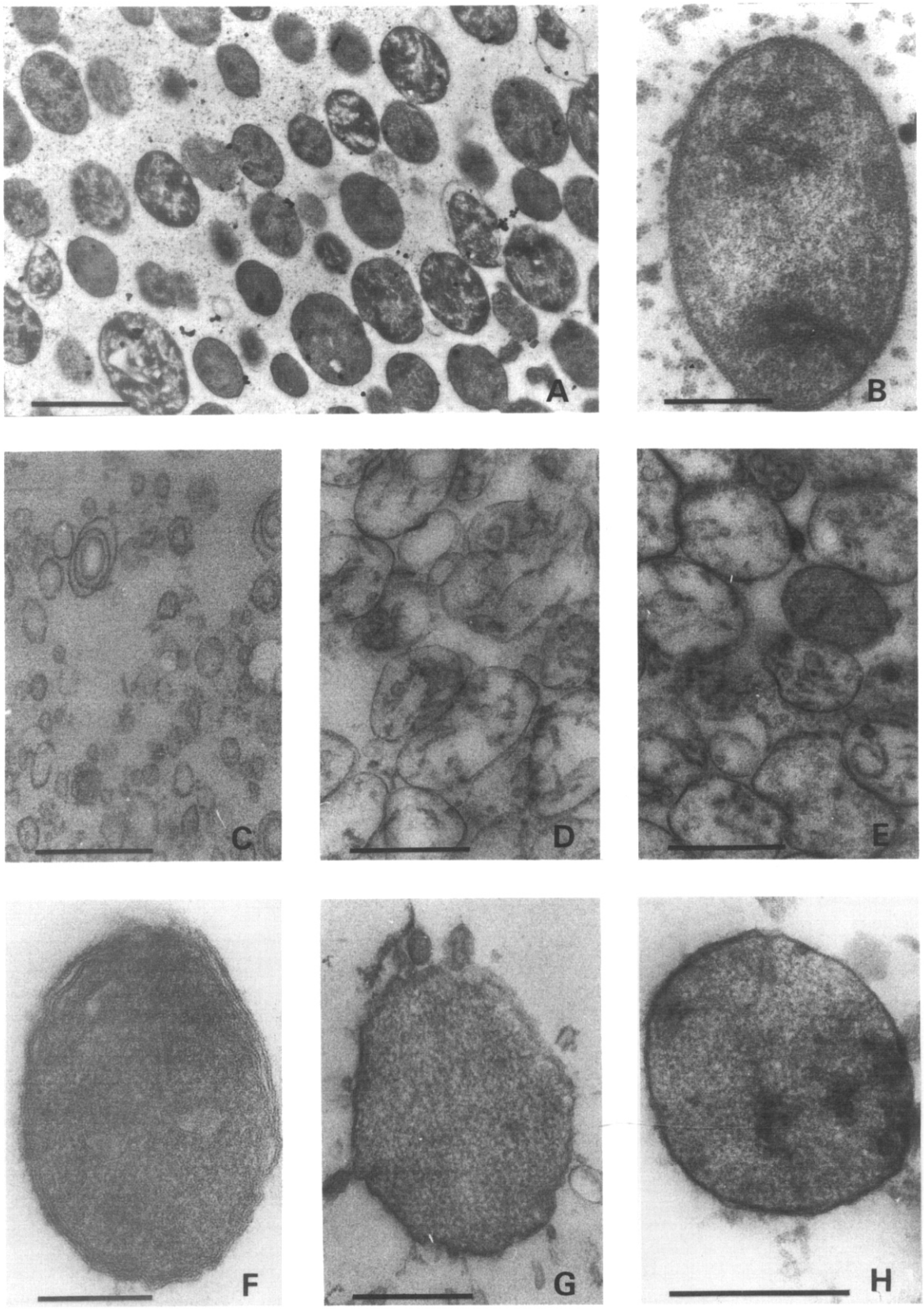

Fig. 1. Electronmicrographs obtained after freeze substitution and low-temperature embedding. Isolated rat liver mitochondria show an orthodox configuration (A) with both membranes in close approximation (B). The subfractions obtained by the swell-shrink-sonicate procedure are shown: the outer membrane fraction OM (C), the inner membrane fraction IM (D) and the inner membrane and matrix fraction IMM (E). Representative mitochondria treated with a digitonin/protein ratio of 0.1, 0.2 and 0.3, respectively, are shown (F–H), demonstrating the gradual removal of the outer membrane. Bars represent 1 μm in A, D and E; 0.5 μm in C, G and H and 0.2 μm in B and F.

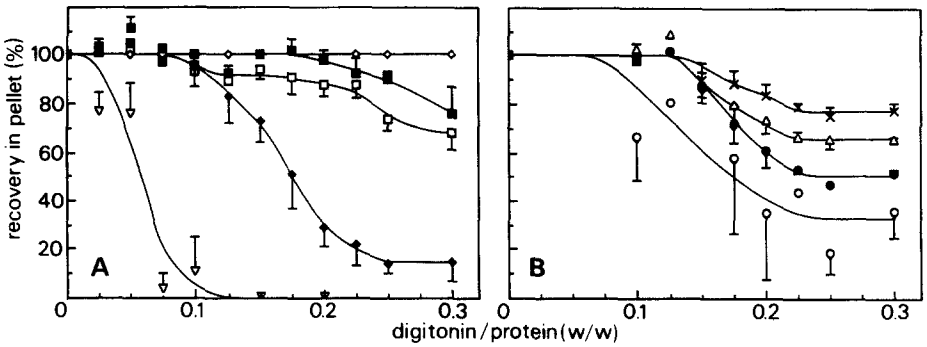

Fig. 2. The effect of digitonin on rat liver mitochondria. Mitochondria were incubated for 15 minutes with the indicated amount of digitonin, whereafter the suspension was diluted and the mitochondrial remnants were pelleted. Marker enzyme activities (A: ◇, cytochrome-*c* oxidase; ■, fumarase; □, protein; ◆, monoamine oxidase; ▽, adenylate kinase) and phospholipids (B: ×, cardiolipin; △, phosphatidylethanolamine (PE); ●, -choline (PC); ○, -inositol (PI) and -serine (PS) of supernatant (now shown) and pellet were determined. For clearity, total phospholipid is not shown. The points and bars represent means ± S.D. of 1–10 experiments.

from the activities of the corresponding marker enzymes. Based on the total succinate–cytochrome-*c* reductase activity, IMM contains (26 ± 17)% and IM (21 ± 12)% of the inner membrane of the subfractionated mitochondria ($n = 17$). Figs. 1D and 1E show that both fractions consist of vesicles of approximately the size of mitochondria and contain tubular, cristae-like structures.

Subfractionation of rat liver mitochondria by the digitonin treatment

An alternative method of choice for separating inner and outer membrane is treating mitochondria with digitonin. Fig. 2 shows the selective and progressive removal of the outer membrane by increasing amounts of digitonin. Roughly, this process can be divided in three parts. Upon increasing the digitonin/protein ratio from

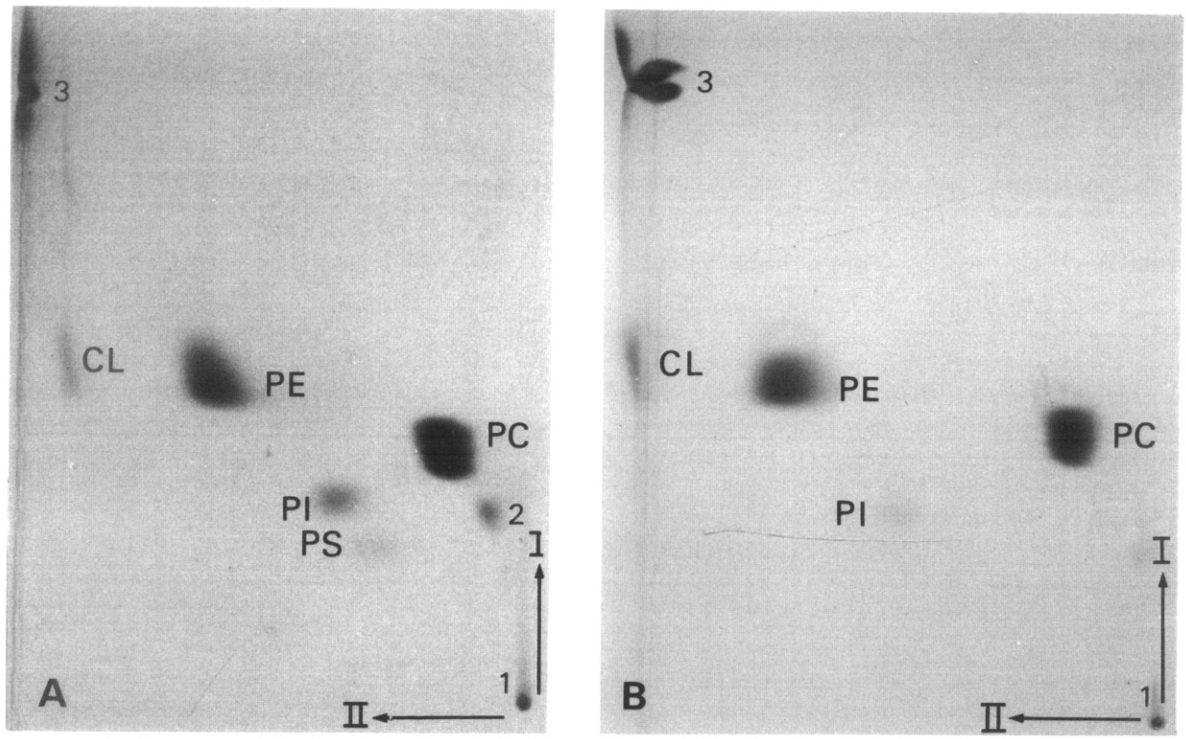

Fig. 3. Two-dimensional thin-layer chromatograms of lipid extracts of OM (A) and IMM (B). Lipids were extracted as described under Methods. Plates were first developed in chloroform/methanol/25% ammonia/water (90 : 54 : 5.5 : 5.5, by vol.) (I) and secondly in chloroform/methanol/acetic acid/water (90 : 40 : 12 : 2, by vol.) (II) [18]. Lipids were detected by iodine vapour. The following spots were identified: PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; CL, cardiolipin. Other symbols, 1, origin; 2, mixture of sphingomyelin, lysophosphatidylcholine and phosphatidylinositol phosphate; 3, unidentified. It should be noted that spots 1 and 3 did not contain phosphate, and spot 2 contained 1–2% of the total lipid phosphorus.

0 to 0.1 the outer membrane becomes permeable to the inter membranous space enzyme adenylate kinase, without significant removal of the outer membrane, as is shown by the almost quantitative recovery of monoamine oxidase (Fig. 2A) and the phospholipids in the pellet (Fig. 2B). Then, by increasing the digitonin/protein ratio from 0.1 to 0.25 the outer membrane is gradually removed as demonstrated by the loss of 85% of the monoamine oxidase and a 41% decrease in the amount of phospholipids recovered in the pellet. Of the mitochondrial phosphatidylserine and -inositol, -choline, -ethanolamine and cardiolipin 72, 50, 34 and 23%, respectively, end up in the supernatant, which is in accordance with the phospholipid compositions of the inner and outer membrane (see forthcoming results). Only when the removal of the outer membrane is almost complete, the inner membrane becomes permeable to the matrix marker fumarase, and respiration ceases to be coupled to phosphorylation (data not shown). Raising the digitonin/protein ratio from 0.25 to 0.3 does not result in changes in the amounts of phospholipid recovered in the pellet. Since cytochrome-*c* oxidase is still quantitatively present in the pellet under these conditions, this suggests that the complete inner membrane is present in the pellet. Therefore, at these digitonin/protein ratios, we call the pellet IM-DIGI and the supernatant OM-DIGI. However, some 15% of the total monoamine oxidase activity remains associated with the pellet. Total phospholipid and marker enzyme activities were quantitatively recovered in the pellet and supernatant.

The progressive removal of the outer membrane was also evident from electronmicroscopy. Fig. 1F shows a mitochondrion, treated with a digitonin/protein ratio of 0.1, of which only the outer membrane has been damaged. At twice the amount of digitonin a partial removal of the outer membrane was observed (Fig. 1G). Mitoplasts devoid of outer membrane were obtained at a ratio of 0.3 (Fig. 1H).

Lipid composition of rat liver mitochondria and subfractions

The phospholipid composition of the various membrane preparations was determined by the use of one- and two-dimensional thin-layer chromatography. As an example, Fig. 3A and B show two-dimensional thin-layer chromatographs of lipid extracts of an outer membrane and an inner membrane plus matrix preparation, respectively.

Table II shows the phospholipid content and composition of our mitochondrial preparation. The phospholipid content of 230 nmol lipid phosphorus per mg protein corresponds well with published values, as well as the overall phospholipid composition [1,40,41]. The major phospholipids are the zwitterionic phosphatidylcholine and -ethanolamine which are present in almost equal amounts. Cardiolipin (18.5%) is the most abundant acidic phospholipid and phosphatidylinositol appears to be more abundant than -serine. In addition, some lipid phosphorus (1–2%) is present as sphingomyelin, phosphatidylinositol phosphate and lysolipids. When cardiolipin is taken as a mitochondrial marker, the isolated preparation is enriched (2.6 ± 0.5-fold relative to the homogenate ($n = 2$). Correction for the lipid compositions of contaminating plasma membrane [1], lysosomes [1], peroxisomes ([33] and D. Hardeman, personal communication) and microsomes, comprising approx. 11.5% of the total phospholipid in this preparation, leads to a 4% decrease in the phosphatidylcholine and a 1.5% increase in the cardiolipin content, while the total phospholipid content is unaffected.

The fractions obtained by the swell-shrink-sonicate procedure contain approximately 40% of the phospholipid loaded on the gradients; the remainder was recovered in the gradients outside the bands, from which these fractions were collected and in the supernatants of the washings of the final pellets. The outer membrane fraction OM displays several striking features: a very high phospholipid to protein ratio and a sizable content of cardiolipin (Fig. 3A). Furthermore, it contains considerably more phosphatidylcholine than -ethanolamine. The sum of phosphatidylinositol and -serine is increased 2.2-times in this fraction relative to the mitochondria and the ratio phosphatidylinositol to -serine is 10. Correcting for the lipid compositions of contaminating plasma membrane [1], lysosomes [1], inner membrane and microsomes, which make up approx. 15% of the phospholipid in OM, leads to an increase in the phospholipid content to approx. 1500 nmol lipid phosphorus per mg protein, without changing significantly the relative amounts of the various phospholipid species.

The phospholipid contents of the inner membrane fractions IM and IMM are intermediate between those of intact mitochondria and the outer membrane fraction OM. They are slightly enriched in phosphatidylethanolamine relative to -choline and contain almost no phosphatidylinositol and -serine (Fig. 3B). The most striking feature of these fractions is the cardiolipin content of only 12 to 14%, being much less than expected, both judged on literature data [1,34] and the composition found for IM-DIGI. This could be due to the non quantitative yield of this subfractionation method and/or to the presence of domains in the inner membrane with different lipid compositions [7,35]. When we take into account the contamination with outer membrane, corresponding to approx. 10% of the phospholipid, it can be concluded that virtually all phosphatidylinositol and -serine present in these fractions originate from the outer membrane.

Lipid analysis of OM-DIGI and IM-DIGI showed that OM-DIGI contained (41 ± 3), (23 ± 3), (34 ± 2),

(50.5 ± 0.7) and (73 ± 7)% of the total mitochondrial phospholipids, total cardiolipin, phosphatidylethanolamine, -choline and -inositol plus -serine, respectively ($n = 4$). The lipid composition of OM-DIGI compared very well with that of OM. Again a considerable amount of cardiolipin was found in this outer membrane fraction. Analyzing the lipids of IM-DIGI, we found that this contained more phosphatidylethanolamine than -choline and little phosphatidylinositol and -serine, like IM and IMM. But unlike IMM and IM this fraction exhibited the expected high content of cardiolipin. This difference might be explained by the fact that the digitonin procedure causes no or little damage to the inner membrane and leads to quantitative yields.

Discussion

This study describes a new procedure to isolate rat liver mitochondria, subfractionation of these mitochondria via two independent and newly adopted methods and the phospholipid composition and content of the mitochondria and the subfractions. It also presents, for the first time, the effects of increasing amounts of digitonin on the release of phospholipids from mitochondria.

The mitochondria are of high purity and quality, as is shown by marker enzyme analysis, the well coupled respiration and the morphology in electronmicrographs of freeze substituted samples. The purity of the mitochondria compares favourably to that achieved by methods, which make use of differential centrifugation [36,37], Ficoll gradients [38] or a different Percoll gradient [39]. However, the yield, based on the total activity of cytochrome-*c* oxidase, of our method is 2–3.5-times less than that of the other methods [38,39]. Hence, the described method favours purity over yield. The extent of intactness of the outer membrane, measured by the latency of cytochrome-*c* oxidase, agrees well with data published on Percoll purified potato tuber mitochondria [40].

Functionality of the isolated mitochondria was also demonstrated by their ability to efficiently import and process an artifical mitochondrial precursor protein, consisting of the first 22 amino acids of the presequence of subunit IV of cytochrome-*c* oxidase linked to mouse cytosolic dihydrofolate reductase (data not shown), which had been shown to be imported and processed by yeast mitochondria previously [41].

The adopted swell-shrink-sonicate procedure described here yields inner and outer membrane preparations which are more enriched in the corresponding marker enzyme than reported in [4], using only hypotonic swelling, and [5,42], employing a different swell-shrink-sonicate procedure. Comparable efficient inner and outer membrane separation was obtained in Ref. 34, using the method of Ref. 4. The yield of outer membrane, based on the recovery of monoamine oxidase activity, for our method is 21%, which is 1.3–3.3-times less than in these other studies [5,43]. The yield of inner membrane is comparable to the one reported in Ref. 5.

Contamination by plasma membrane, microsomes, lysosomes and peroxysomes in the outer membrane fraction OM, based on the specific marker enzyme activities relative to the mitochondria, reported here is higher than in Ref. 34. However, based on the specific activity of glucose-6-phosphatase relative to isolated microsomes our preparation contains less microsomes. The extent of microsomal contamination, based on the glucose-6-phosphatase activity probably is an overestimation due to unspecific phosphatase reactions [42].

Gradual and selective release of marker enzymes from mitochondria by increasing amounts of digitonin was achieved by the described method using relatively low mitochondrial protein concentrations. Developing our digitonin extraction method, we noticed that under different experimental conditions it was possible to solubilize all phospholipid species to the same extent even at low digitonin/protein ratios (data not shown), indicating that both inner and outer membrane were affected at the same time. The extraction procedure is superior to methods using protein concentrations of 50 mg/ml during incubation [6,44–46] and methods using lower protein concentrations (10 or 5 mg/ml) [46,47], where digitonin already causes severe damage to the inner membrane when the outer membrane is not completely removed yet. With those methods the removal of inter membrane space components, outer membrane and matrix components are less well separated, sequential events. It is shown (Fig. 2) that the phospholipids from the outer membrane were released in close parallel to monoamine oxidase. The residual monoamine oxidase activity of 15% is higher than the 5% reported in Refs. 44 and 46 and the 0% in Refs. 6 and 45. The removal of monoamine oxidase by increasing amounts of digitonin is parallelled by the release of porin and glutathione transferase, as determined by Western blotting with anti-porin and anti-outer membrane antisera, respectively (data not shown), confirming data in Ref. 46 on porin and monoamine oxidase. Both glutathione transferase and part of the porin are thought to be constituents of contact sites [48]; thereby suggesting that the digitonin treatment does not discriminate between outer membrane in- and outside contact sites.

The lipid composition of our mitochondria and the subfractions, obtained by the swell-shrink-sonicate procedure and the digitonin treatment, correspond well with data in Ref. 34. These data show a cardiolipin content of the inner membrane fractions, obtained by the hypotonic swelling method, which is lower than expected, be it not as extreme as what we found (Table III); for this we have no solid explanation. The content of phosphatidylinositol and -serine in the inner mem-

TABLE III

Lipid composition of rat liver mitochondria and subfractions obtained by the swell-shrink-sonicate procedure and the digitonin treatment

Data are presented as means ± S.D. and the number of experiments is given in parenthesis. Phospholipid compositions are expressed as percentages of total lipid phosphorus. * Not in all experiments phosphatidylinositol and -serine were separated; this row also includes the values of the experiments in which phosphatidylinositol and -serine were determined separately. n.d., not determined.

	Mitochondria	OM	IMM	IM	OM-DIGI	IM-DIGI
nmol phospholipid/mg protein	230 ±30 (8)	1110 ±150 (4)	340 ±110 (6)	450 ±90 (6)	n.d.	n.d.
Phosphatidylcholine	39 ± 3 (13)	48 ± 5 (7)	39 ± 4 (6)	39.2± 0.9 (6)	50±7 (4)	33 ±3 (4)
Phosphatidylethanolamine	37.0± 0.7 (13)	31.0± 1.6 (6)	44.6± 0.9 (5)	43.4± 1.1 (7)	32±4 (4)	41 ±3 (4)
Cardiolipin	19 ± 3 (13)	9 ± 2 (4)	11 ± 2 (6)	13.7± 2.3 (7)	11±2 (4)	24 ±4 (4)
Phosphatidylinositol	2.4± 1.6 (2)	9.9± 1.7 (4)	0.7± 0.2 (2)	1.0± 0.2 (2)	n.d.	n.d.
Phosphatidylserine	0.8± 0.6 (2)	1.0± 0.3 (3)	0.4 (1)	0 ± 0 (2)	n.d.	n.d.
Phosphatidylinositol + -serine *	4 ± 2 (13)	8.9± 1.8 (6)	1.5± 0.9 (7)	1.1± 0.8 (7)	7±4 (4)	1.9±1.0 (4)

brane fractions, obtained by our method can completely be attributed to contamination with outer membranes. Many data on lipid composition and content of mitochondria and subfractions have been published, which deviate from our data; to what extent these differing results are due to the used isolation procedures is not clear, because no data on marker enzymes are reported in these studies [49–52]. 23% of the total mitochondrial cardiolipin is found to be present in the outer membrane, which is not due to inner membrane contaminations. Cardiolipin, a unique mitochondrial lipid, is not only very important in the inner membrane, where it is thought to be important for the functioning of several enzymes [53,54], but may also play a prominent role in the outer membrane in the import of mitochondrial precursor proteins as was suggested by recent studies [55,56].

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References

1 Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42.
2 Vance, J.E. (1988) Biochim. Biophys. Acta 963, 10–20.
3 Parsons, D.F. and Verboon, J.G. (1965) J. Applied Phys. 36, 2615–2616.
4 Parsons, D.F., Williams, G.R. and Chance, B. (1966) Ann. N.Y. Acad. Sci. 137, 643–666.
5 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438.
6 Schnaitman, C. and Greenawalt, J.W. (1968) J. Biol. Chem. 38, 158–175.
7 Brdiczka, D., Dölken, G., Krebs, W. and Hofmann, D. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 731–743.
8 Weissbach, H., Smith, T.E., Daly, J.W., Witkop, B. and Udenfriend, S. (1960) J. Biol. Chem. 235, 1160–1163.
9 Bergmeyer, H.U. (1970) Methoden der enzymatischen Analyse, pp. 457–458, Verlag Chemie, Weinheim.
10 Holmes, R.S. and Masters, C.J. (1970) FEBS Lett. 11, 45–48.
11 Emmelot, P., Bos, C.J., Benedetti, E.L. and Rümke, P.H. (1964) Biochim. Biophys. Acta 90, 126–145.
12 Schmidt, B., Wachter, E., Sebald, W. and Neupert, W. (1984) Eur. J. Biochem. 144, 581–588.
13 Kanarek, L. and Hill, R.L. (1964) J. Biol. Chem. 239, 4202–4206.
14 Swanson, M.A. (1955) Methods Enzymol. 2, 541–543.
15 Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 409–427.
16 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–47.
17 Bligh, E.G. and Dyer, W.J. (1959) J. Biol. Chem. 37, 911–917.
18 Broekhuijse, R.M. (1969) Clin. Chim. Acta 23, 457–461.
19 Fiske, L.M. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–389.
20 Voorhout, W.F., Leunissen-Bijvelt, J.J.M., Van der Krift, Th.P. and Verkleij, A.J. (1990) Scanning, in press.
21 Millonig, G. (1961) J. Biophys. Biochem. Cytol. 11, 736–739.
22 Pertoft, H., Laurent, T.C., Låås, T. and Kågedal, L. (1978) Anal. Biochem. 88, 271–282.
23 Goglia, F., Liverini, G., Lanni, A., Iossa, S. and Bartella, A. (1988) Biochem. Biophys. Res. Commun. 151, 1241–1249.
24 Wu, M. and Tzagoloff, A. (1987) J. Biol. Chem. 262, 12275–12282.
25 Loten, E.G. and Redshaw-Loten, J.C. (1986) Anal. Biochem. 154, 183–185.
26 Ghosh, M.K. and Hajra, A.K. (1986) Anal. Biochem. 159, 169–174.
27 Rupar, C.A. and Whitehall, J.D. (1988) Biochem. Cell Biol. 66, 273–278.
28 Nakabayashi, T. and Ikezawa, H. (1988) Biochem. Int. 16, 1119–1125.
29 Johnson, D. and Lardy, H. (1967) Methods Enzymol. 10, 94–96.
30 Malhotra, S.K. and Van Harreveld, A. (1965) J. Ultrastruct. Res. 12, 473–487.
31 Knoll, G. and Brdiczka, D. (1983) Biochim. Biophys. Acta 733, 102–110.
32 Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Biochim. Biophys. Acta 860, 672–689.
33 Lazarow, P.B. (1984) in Membrane Structure and Function (Bittar, E.E., ed.), pp. 1–33, John Wiley and Sons, New York.
34 Colbeau, A., Nachbaur, J. and Vignais, P.M. (1971) Biochim. Biophys. Acta 249, 462–492.
35 Werner, S. and Neupert, W. (1972) Eur. J. Biochem. 25, 379–396.
36 Beuafay, H. and Amar-Costesec, A. (1976) Meth. Membr. Biol. 6, 1–100.

37 Bustamante, E., Soper, J.W. and Pedersen, P.L. (1977) Anal. Biochem. 80, 401–408.
38 Stocco, D.M. (1983) Anal. Biochem. 131, 453–457.
39 Reinhart, P.H., Taylor, W.M. and Bygrave, F.L. (1982) Biochem. J. 204, 731–735.
40 Neuburger, M., Journet, E., Bligny, R., Carde, J. and Douce, R. (1982) Arch. Biochem. Biophys. 217, 312–323.
41 Vestweber, D. and Schatz, G. (1989) Nature 338, 170–172.
42 Martinez, P. and McCauley, R. (1977) Biochim. Biophys. Acta 497, 437–446.
43 Murthy, M.S.R. and Pande, S.V. (1987) Proc. Natl. Acad. Sci. USA 84, 378–382.
44 Hoppel, C. and Cooper, C. (1968) Biochem. J. 107, 367–375.
45 Zammit, V.A. and Corstorphine, C.G. (1985) Biochem. J. 230, 389–394.
46 Jancsik, V., Lindén, M., Dorbani, L., Rendon, A. and Nelson, B.D. (1988) Arch. Biochem. Biophys. 264, 295–301.
47 Matlib, M.A. and O'Brien, P.J. (1975) Arch. Biochem. Biophys. 167, 193–202.
48 Krause, J., Hay, R., Kowollik, C. and Brdiczka, D. (1986) Biochim. Biophys. Acta 860, 690–698.
49 Canuto, R.A., Biocca, M.E., Muzin, G. and Dianzani, M.U. (1989) Cell. Biochem. Funct. 7, 11–19.
50 Ruggiero, F.M., Landriscina, C., Gnoni, G.V. and Quagliariello, E. (1984) Lipids 19, 171–178.
51 Guarnieri, M., Stechmiller, B. and Lehninger, A. (1971) J. Biol. Chem. 246, 7526–7532.
52 Ishankhodzhaev, T.M., Bornikov, V.T., Zainutdinov, B.R. and Saatov, T.S. (1987) Biokhimiya 52, 220–224 (Eng).
53 Fry, M. and Green, D.E. (1980) Biochem. Biophys. Res. Commun. 93, 1238–1246.
54 Kadenbach, B., Mende, P., Kolbe, H.V.J., Stipani, F. and Palmieri, F. (1982) FEBS Lett. 139, 109–112.
55 Ou, W., Ito, A., Umeda, M., Inoue, K. and Omura, T. (1988) J. Biochem. 103, 589–595.
56 Eilers, M., Endo, T. and Schatz, G. (1989) J. Biol. Chem. 264, 2945–2950.