BIOCHEMISTRY AND BIOPHYSICS OF MITOCHONDRIAL MEMBRANES

Edited by

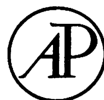
G. F. AZZONE
Istituto di Patologia Generale e Centro per lo Studio della Fisiologia dei Mitocondri del CNR Universita di Padova, Padova, Italy

E. CARAFOLI
Istituto di Patologia Generale, Universita di Modena Modena, Italy

A. L. LEHNINGER
Department of Physiological Chemistry The Johns Hopkins University School of Medicine Baltimore, Maryland

E. QUAGLIARIELLO
Istituto di Chimica Biologica e Centro di Studio sui Mitocondri e Metabolismo Energetico del CNR Universita di Bari, Bari, Italy

N. SILIPRANDI
Istituto di Chimica Biologica e Centro per lo Studio della Fisiologia dei Mitocondri del CNR Universita di Padova, Padova, Italy



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STRUCTURE AND BIOGENESIS OF OUTER AND INNER MITOCHONDRIAL MEMBRANES OF NEUROSPORA CRASSA

Walter Neupert, George D. Ludwig,† and A. Pfaller

Institut fur Physiologische Chemie und
Physikalische Biochemie der Universitat Munchen
8000 Munchen 15, Goethestrasse 33
German Federal Republic

INTRODUCTION

Abundant evidence has been presented which indicates that two protein-synthesizing systems cooperate in mitochondrial biogenesis, one localized within the mitochondria (intrinsic) and the other outside the mitochondria in the cytoplasm (extrinsic). It is widely held that both systems contribute to the biogenesis of mitochondrial membrane proteins, while the soluble matrix proteins are synthesized exclusively by the extrinsic system (1). The mitochondrial membrane system can be separated into outer and inner membranes (2). From studies performed principally on liver mitochondria it is obvious that these two membranes are quite different in structure and function (2). On the basis of these differences and the different response of the synthesis of the membranes to antibiotics, it has been suggested that the outer mitochondrial membrane is produced by the extrinsic system (3-5). In support of this hypothesis, it has been shown that isolated rat liver mitochondria incorporate labeled amino acids into the inner membrane proteins only, implying that outer membrane is produced by the cytoplasmic system (6-8). However, *in vitro* protein synthesis in isolated mitochondria is potentially subject to a number of defects. Therefore, a study was made with *Neurospora crassa* so that mitochondrial membrane protein synthesis could be investigated in whole cells, utilizing cycloheximide as a specific inhibitor of cytoplasmic protein synthesis (9-11).

A simultaneous study of the structure of Neurospora

† Footnote

outer and inner mitochondrial membranes was begun to gain more insight into the biogenesis of individual membrane components.

METHODS

Hyphae of Neurospora crassa (wild type 74A) were grown under sterile conditions for 18-20 hours at 25°C in 8 liter bottles which were continuously aerated. The hyphae were harvested, filtered, washed, homogenized briefly and the cells finally disrupted in a grinding mill. The mitochondria were then isolated by differential centrifugation.

A modified combined swelling - shrinking - sonication procedure described by Parsons et al (3, 12) and by Sottocasa et al (4), and first applied to Neurospora mitochondria by Cassady (13, 14), was used to detach outer from inner membrane. The membranes were then separated by centrifugation through a 50 ml. linear sucrose density gradient varying between 0.95 and 1.5 M sucrose in 10 mM Tris - HCl, pH 7.5, for 1 hour at 0°C and 75,000 X g in a Spinco ultracentrifuge. The gradient was cut into 11 fractions of 5 ml., each of which was diluted in Tris - HCl and centrifuged at 151,000 X g for 1 hour. The pellets were resuspended in potassium phosphate buffer, again centrifuged, and finally resuspended to a protein concentration of 2-5 mg./ml. in 0.1 M potassium phosphate buffer, pH 7.2.

Since outer membrane of Neurospora mitochondria has been shown to lack monamine oxidase and rotenone - insensitive NADH - cytochrome c reductase activity (14), kynurenine hydroxylase (KH) was used as a marker enzyme for outer membrane. KH was measured by the method of Schott et al (15). Succinate - cytochrome c reductase as well as cytochrome concentrations were used as inner membrane markers. Succinate - cytochrome c reductase was measured according to the method of Arrigoni and Singer (16) with addition of phenazine methosulfate. Cytochrome concentrations were calculated from difference spectra recorded in a split beam spectrophotometer.

Phospholipid phosphorus was estimated according to Folch et al (17). For ergosterol determinations membrane preparations were extracted with methanol and acetone. After transfer into hexane, UV spectra were recorded and ergosterol content was calculated from the extinction at

282 nm.

RESULTS

Submission of mitochondria to a swelling - shrinking - sonication procedure, followed by centrifugation through a linear density gradient, distributes most of the protein in the lower third of the tube as a dark brown band (fractions 6-9, Fig. 1A). At the interface between the volume initially applied (fraction 1) and the top of the density gradient, a red orange band is seen, which is collected in fraction 2. This band is absent if mitochondria are applied directly to the gradient without previous swelling - shrinking - sonication. Fractions 1 and 2 contain 2-4% of the total protein. The amount of material recovered in fraction 1 depends upon the intensity of sonication. Fraction 1 was not examined in cases in which it was less than 10% of fraction 2 on a protein basis.

Kynurenine hydroxylase activity, which serves as an outer membrane marker (13, 14, 18), is concentrated in fractions 1 and 2 and low in other fractions (Fig. 1B). Similar results are obtained for a carotenoid pigment, which serves as an additional outer membrane marker (Fig. 1C). The latter pigment is readily extracted by methanol and acetone from the red sediments obtained by centrifugation of fractions 1 and 2. It can then be transferred into hexane. The pigment is acidic and possesses a carotenoid-like absorption spectrum with maximum absorption at 477 nm and shoulders at 450 and 510 nm. From its solubility characteristics in the various solvents and its absorption spectrum, the pigment has been identified as neurosporaxanthine, previously demonstrated to be the main acidic carotenoid of Neurospora crassa (19, 20). Spectral analysis showed that neurosporaxanthine is the only carotenoid occurring in mitochondria. In several experiments 60-80% of the total neurosporaxanthine in the hyphae was found to be localized in the mitochondria. Taking into account the partial loss of outer membrane from whole mitochondria during isolation, it can be concluded that the vast majority of cellular neurosporaxanthine is located in the outer mitochondrial membrane.

In contrast to kynurenine hydroxylase activity and the carotenoid pigment concentration, succinate-cytochrome c reductase and cytochrome pigments, which serve as inner membrane markers (3, 4, 21), are concentrated in fractions

7-9 of the gradient and are low in fractions 1 and 2. (Fig. 2A and B). The distribution of RNA in the gradient follows a pattern similar to succinate - cytochrome c reductase and the cytochromes (Fig. 2C) permitting the conclusion that outer membrane prepared in this fashion contains neither RNA nor ribosomes.

Electron micrographs were made of samples from all steps in the procedure and from each of the eleven fractions obtained from the sucrose density gradient. Representative samples of positive staining are given in Fig. 3. Fraction 1 (Fig. 3A), as well as fraction 2, (outer membrane), consists almost exclusively of various sized single-membrane vesicles. Fraction 9 (Fig. 3B) (inner membrane) is made up of larger membrane vesicles with some remaining profiles of cristae.

Negatively stained preparations are shown in Fig. 4. Outer membrane (Fig. 4A) displays thick membrane contours and characteristic long rope-like extrusions. Inner membrane (Fig. 4B) has a sharper and thinner membrane contour and no extrusions can be seen.

Polyacrylamide gel electrophoresis of proteins from outer and inner membrane preparations was carried out in a phenol/acetic acid/water system (23) and in a sodium dodecylsulfate system (24). Fig. 5 shows a densitometer tracing of the electrophoretic pattern obtained with the first system after staining protein bands with amido black. Fraction 1 (outer membrane) yields essentially a single protein band which corresponds to band number 11 of whole mitochondria as defined by Sebald et al. (23). Traces of band 4, 5, 6, and 8, which are evident, can be ascribed to contamination by inner membrane. This contamination varied in different experiments between 5 and 20%. Fraction 9 (inner membrane) exhibits all the protein bands that are obtained by electrophoresis of whole mitochondrial membranes, also shown in this graph, except that band 11 is much weaker.

On gels containing 0.1% sodium dodecylsulfate outer membrane also displays essentially one band. The molecular weight corresponding to this band is calculated to be $30{,}000 \pm 1{,}000$ daltons.

The phospholipid contents of the different fractions of the gradient, expressed at μg phospholipid phosphorus per mg insoluble membrane protein is presented in Fig. 6. Clearly, the phospholipid concentration is very high in the outer membrane, while it is much lower in inner membrane.

On the basis of 1 μg phospholipid phosphorus corresponding to 25 μg phospholipid the following values are calculated: outer membrane 1.1 mg and inner membrane 0.25 - 0.3 mg per mg membrane protein.

Besides phospholipid, the outer membrane contains large amounts of ergosterol, varying in different preparations between 200 - 300 μg / mg protein. In inner membrane ergosterol is present in only minute amounts.

The densities of the two mitochondrial membranes as determined by isopycnic centrifugation are approximately 1.07 - 1.1 for outer membrane and 1.22 - 1.25 for inner membrane.

Crystals are formed from pellets or concentrated suspensions of outer membrane preparations when they are permitted to remain standing in the cold or at room temperature. These crystals were identified as ergosterol. Figure 7 shows a phase contrast microscopic picture of such an aged preparation. Ergosterol appears as long curved needles. During the process of aging, outer membrane undergoes an impressive change in structure as shown by the electron microscope (Fig. 8). Large, partly concentric membrane systems are formed. The new vesicles may have a membrane contour length of up to 0.1 mm.

LABELING EXPERIMENTS

Isolated mitochondria were incubated with L-(^{14}C) leucine, L-(^{14}C) isoleucine and L-(^{14}C) phenylalanine under conditions previously determined to be optimal for incorporation of radioactive amino acids (23). After incubation, the swelling - shrinking - sonication procedure was applied to the mitochondria, followed by density gradient centrifugation. The specific radioactivities of the different fractions are shown in Fig. 9. The specific radioactivities of fractions 1 and 2 from the top of the gradient, representing outer membrane, are low compared to those of the inner membrane fractions, indicating that polypeptide chains synthesized by mitochondrial ribosomes in mitochondria isolated *in vitro* are incorporated into inner but not into outer membrane.

To study the biosynthesis of outer membrane proteins in whole cells, two batches of *Neurospora* cells were incubated separately, one with ^{14}C-labeled amino acids without cycloheximide, and the other with ^{3}H-labeled amino acids in the presence of cycloheximide. After labeling, both

portions were combined and outer and inner membranes were prepared. As shown in Fig. 10, in the control experiment (^{14}C-radioactivity), approximately equal specific radioactivities are exhibited by all fractions from the gradient, indicating that outer and inner mitochondrial membrane proteins are synthesized at a similar rate in exponentially growing Neurospora cells.

Cycloheximide has been shown to inhibit cytoplasmic protein synthesis about 99% (10). The specific activity corresponding to this 1% of the control was measured and subtracted from all fractions 1-11. As shown in Fig. 10B, cycloheximide inhibits incorporation into outer membrane (^{3}H radioactivity) much more than it does incorporation into inner membrane fractions. Specific radioactivity and succinate-cytochrome c reductase activity have similar distributions in the gradient. This is reflected in the ratio radioactivity/succinate cytochrome c reductase activity. (Fig. 10B, dashed curve). This ratio is very similar in fractions 1-2 and 8-9, indicating that contamination by inner membrane accounts for the radioactivity present in the outer membrane fraction. The ratio radioactivity/ cytochrome c reductase is very different in the control experiment (Fig. 10A, dashed curve). These observations indicate that labeling of outer membrane protein in the presence of cycloheximide is less than 2-3% of the labeling of the inner membrane protein.

DISCUSSIONS AND CONCLUSIONS

The results obtained demonstrate that the combined swelling - shrinking - sonication procedure developed by Parsons et al (3) and Sottocasa et al (4) for liver mitochondria is applicable to Neurospora mitochondria, confirming the work of Cassady (13, 14, 25). Kynurenine hydroxylase activity is shown to be localized in the outer membrane of Neurospora crassa, also confirming Cassady (13).

A red carotenoid pigment with spectral and solubility properties characteristic of neurosporaxanthine is also shown to be concentrated in the outer membrane fractions, thus affording an additional satisfactory marker for outer membrane of Neurospora. The function of this carotenoid is unknown. It may be speculated that it serves as a free radical scavenger that may protect the phospholipids in the outer membrane from peroxidations induced by light, or

that it may absorb light preventing damage to mitochondrial inner membrane (26). Very low concentrations of carotenoid are found in Neurospora grown in the dark and the formation of carotenoids in Neurospora is dependent upon light (19).

The outer membrane preparations from Neurospora mitochondria yield only a single band in electrophoresis on polyacrylamide gel, in contrast to outer membrane from rat liver mitochondria (27). The minor bands found in this study appear to be derived from contaminating inner membrane, as shown both by the electrophoretic studies and marker enzyme activities. The results obtained suggest that greater than 95% of outer membrane protein of Neurospora mitochondria represents either a single protein, multiple proteins with similar mobility, or the capacity to form aggregates under the electrophoretic conditions applied.

Outer membrane preparations from Neurospora show no RNA or ribosomes, in contrast to outer membrane preparations from rat liver mitochondria (7). Moreover, since Neurospora possess no membrane system analagous to the endoplasmic reticulum of liver cells, the outer membrane fractions are not contaminated with microsomes.

The very high phospholipid content of Neurospora outer mitochondrial membranes seems to reflect a general principle of outer membrane structure since similar values have been reported for guinea pig liver (28) and rat liver (29, 30). The large difference in phospholipid content of outer and inner membrane is certainly responsible for the differences in the densities of the membranes. The values reported here are very close to those for guinea pig liver (12).

The occurrence of ergosterol in Neurospora outer mitochondrial membrane would suggest that sterols in general are essential components of outer mitochondrial membranes, since cholesterol has been detected in rather high concentrations in outer membrane of liver mitochondria (28, 29).

The extremely high concentration of ergosterol appears to confer strange physical properties to Neurospora outer mitochondrial membrane. This is indicated by the conversion of small vesicles into very large ones upon aging, accompanied by ergosterol crystallizing out and by the formation of long membrane extrusions in negative staining, probably as an artifact. In the inner membrane the low concentration of ergosterol (about 3% compared to outer

membrane) is probably due to contamination by outer membrane. Thus, it is concluded that inner membrane is virtually devoid of ergosterol. Variable amounts of cholesterol have been reported from preparations of inner membrane from liver mitochondria, namely 16-45% as compared to outer membrane (28, 29). Since, in these experiments, these concentrations were not correlated with marker enzyme activities to correct for contamination by outer membrane, it is not clear whether cholesterol is present in the inner membrane of liver mitochondria at all.

The results of the studies of incorporation of labeled amino acids into isolated Neurospora mitochondria agree with those obtained with rat liver mitochondria (6-8), suggesting that the cytoplasmic extramitochondrial protein - synthesizing system is required for the synthesis of outer membrane protein. Additional confirmation is derived from the studies of whole cells, in vivo, in which cytoplasmic protein synthesis is nearly completely inhibited by cycloheximide, a specific inhibitor of cytoplasmic protein synthesis (9-11). These results provide conclusive evidence that at least 97-98% of outer membrane protein of mitochondria must be synthesized by the extramitochondrial cytoplasmic protein-synthesizing system, while the intrinsic system contributes only to the biogenesis of inner membrane proteins.

A portion of the results described here have been published (31).

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†Footnote
Present address: Department of Medicine, Medical College of Ohio, Toledo, Ohio 42614, USA

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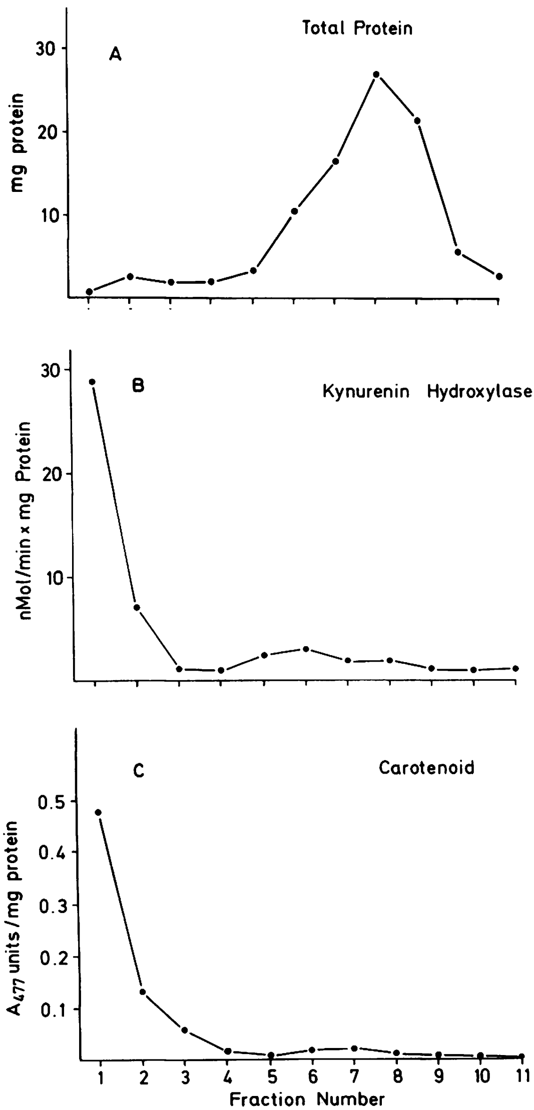

Fig. 1. Distribution of mitochondrial protein (A), kynurenine hydroxylase (B), and carotenoid pigment (C), in a lirear sucrose density gradient through which a mitochondrial preparation was centrifuged after swelling, shrinking and sonication. Fraction 1 represents the top and fraction 11 the bottom of the tube. Each fraction volume is 5 ml.

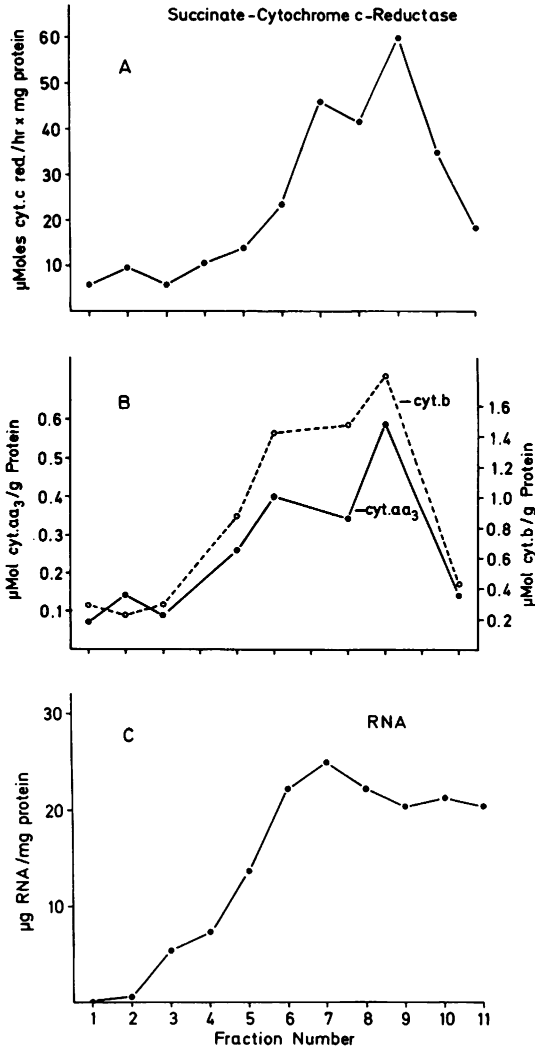

Fig. 2. Distribution in a linear sucrose density gradient of succinate - cytochrome c reductase activity (A), cytochromes aa3 and cytochrome b (B), and RNA concentration (C) from mitochondria previously subjected to swelling, shrinking and sonication, then centrifuged through the gradient. Fractions and volumes are the same as shown in Fig. 1.

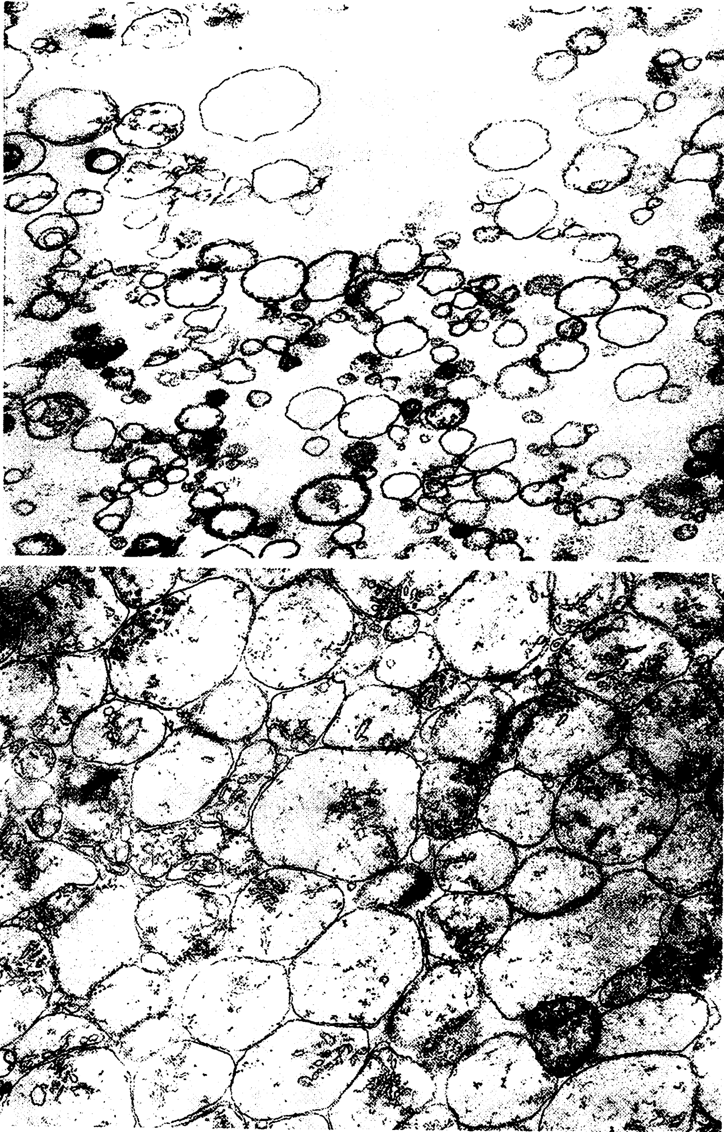

Fig. 3. Electron micrographs of fraction 1 from the gradient (outer membrane preparation), A, above, x 20,000, and of fraction 9 from the gradient (inner membrane preparation), B, below, (x 14,000).

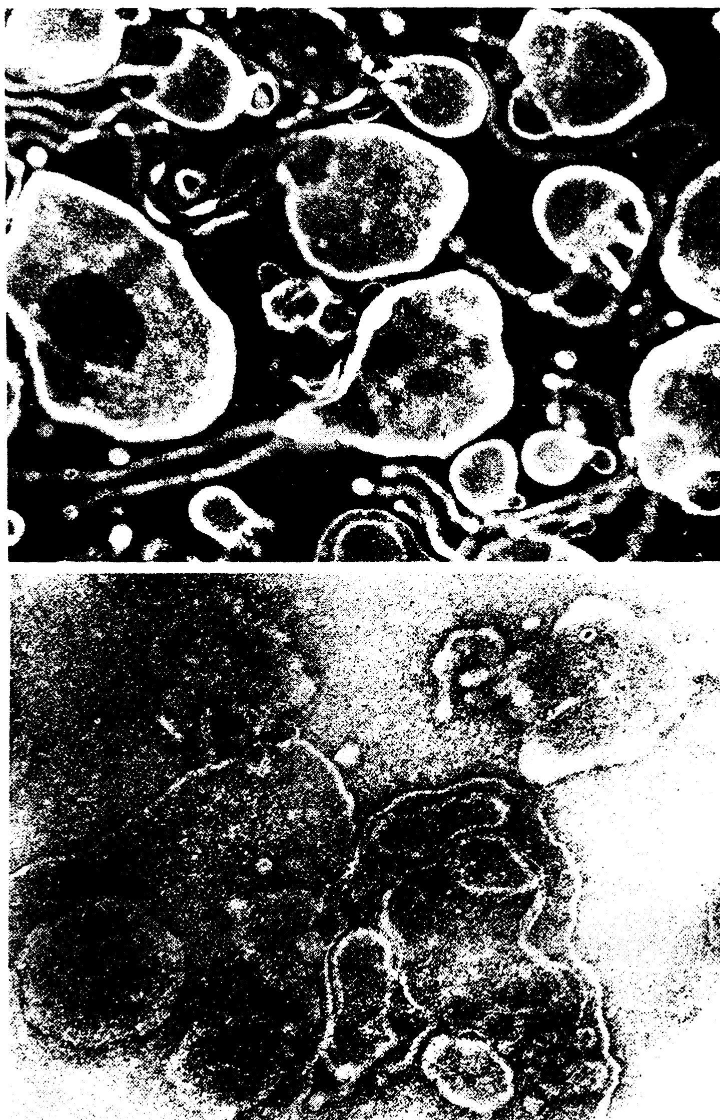

Fig. 4. Negatively stained specimens of fraction 2 (outer membrane preparation), A, above, and of fraction 9 (inner membrane preparation), B, below, x 82,000. Negative staining was performed as described by Parsons (22).

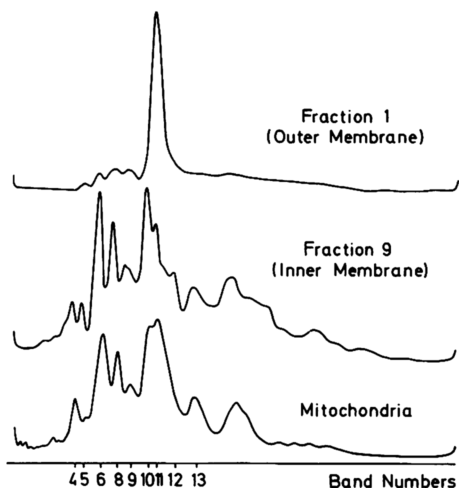

Fig. 5. Polyacrylamide gel electrophoresis of protein from outer (fraction 1) and inner (fraction 9) membrane preparation and of whole mitochondrial membranes. Gels were stained with amido black and densitometry was performed at 546 nm.

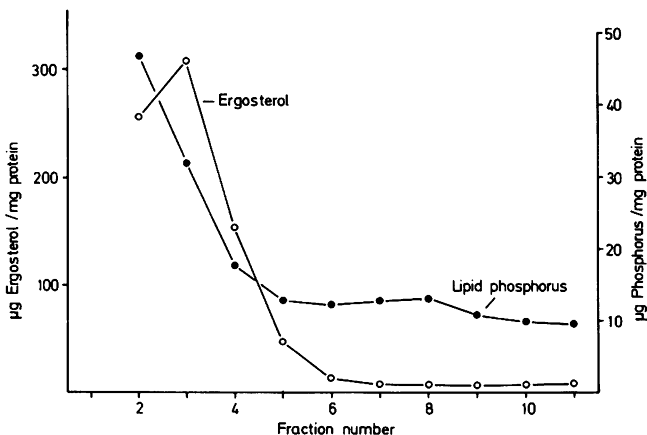

Fig. 6. Distribution of phospholipid phosphorus and of ergosterol in a linear sucrose density gradient through which a mitochondrial preparation was centrifuged after swelling, shrinking and sonication. Fractions and volumes as in Fig. 1. Soluble proteins were extracted from all fractions by sonication in 0.1M phosphate buffer and a successive 60 min. 144,000 x g centrifugation. Fraction 1 was not measured in this case, since only minimal amounts of outer membrane were recovered in this fraction.

Fig. 7. Phase contrast picture of an outer mitochondrial membrane preparation, aged by standing for 5 hours at 20°C. (x 750).

Fig. 8. Electron micrograph (positive staining) of outer mitochondrial membrane after aging 5 hours at 20°C. (x 14,000).

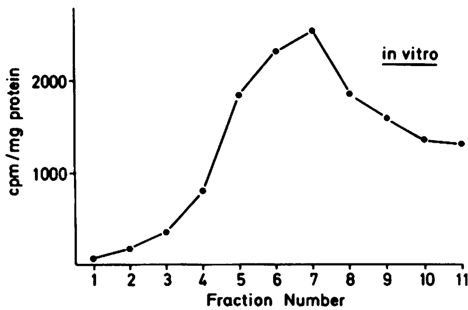

Fig. 9. Specific radioactivities of mitochondrial outer and inner membrane fractions after incorporation of labeled amino acids into isolated mitochondria. Fractions were obtained by swelling, shrinking and sonication and subsequent sucrose density gradient centrifugation. Fractions and volumes as in Fig. 1. For further details see ref. (10).

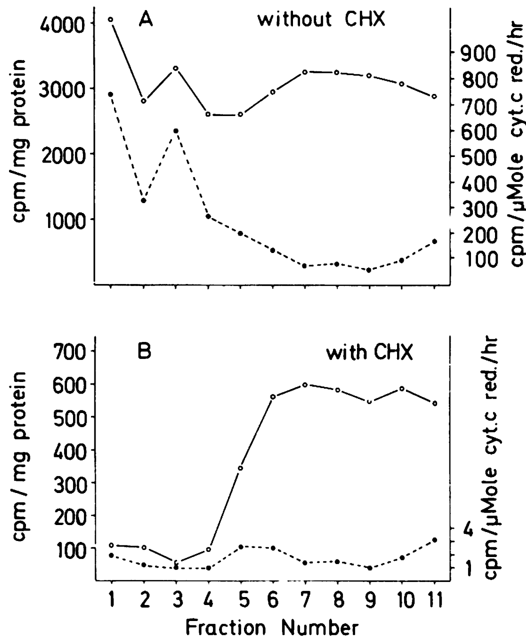

Fig. 10. Incorporation *in vivo* of labeled amino acids into outer and inner mitochondrial membrane fractions in the presence and absence of cycloheximide. Cells were labeled in two separate portions, one with ^{14}C-labeled-amino acids in the absence of cycloheximide, the other with ^{3}H-labeled-amino acids in the presence of 0.1 mg/ml cycloheximide. The two portions were combined and outer and inner mitochondrial membrane fractions were prepared by swelling, shrinking and sonication and density gradient centrifugation. Fraction 1 represents top and fraction 11 bottom of the gradient. (A) Without cycloheximide (^{14}C radioactivity); (B) with cycloheximide (^{3}H radioactivity).

0 = specific radioactivities (counts x min^{-1} x mg $protein^{-1}$);

0 = ratio radioactivity/succinate-cytochrome c reductase activity (counts x min^{-1} per μmoles cytochrome c reduced x h^{-1}).