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Sites of Biosynthesis of Outer and Inner Membrane Proteins of *Neurospora crassa* Mitochondria

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Outer and inner membranes of *Neurospora crassa* mitochondria were separated by the combined swelling, shrinking, sonication procedure. Membranes were characterized by electron microscopy and by marker enzyme activities. A red carotenoid pigment was found to be concentrated in the outer membrane. The inner mitochondrial membrane was resolved into about 20 protein bands on polyacrylamide gel electrophoresis, whereas the outer membrane shows essentially one single protein band. Only negligible incorporation of radioactive amino acids occurs into outer membrane when isolated mitochondria are synthesizing polypeptide chains. In agreement with this observation labeling of outer membrane protein is almost entirely blocked, when whole *Neurospora* cells are incubated with radioactive amino acids in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis. Finally, the essential electrophoretic protein band from outer membrane does not become labeled when mitochondria are incubated with radioactive amino acids either *in vitro* or *in vivo* in the presence of cycloheximide. It is concluded that the vast majority, if not all, of the outer membrane protein is synthesized by the cytoplasmic system and that polypeptide chains formed by the mitochondrial ribosomes are integrated into the inner mitochondrial membrane.

Two systems of protein synthesis, one mitochondrial (intrinsic) and the other extramitochondrial or cytoplasmic (extrinsic) contribute to mitochondrial biogenesis. It is generally accepted that both systems provide proteins to be integrated into the mitochondrial membranes, whereas the soluble matrix proteins of mitochondria are supplied by the extrinsic system [1]. In distinguishing between the biogenesis of mitochondrial outer and inner membranes, it was suggested that the outer membrane of rat liver mitochondria was derived from the endoplasmic reticulum and hence its proteins synthesized by the cytoplasmic system [2,3]. The latter suggestion was substantiated by the observation that isolated mitochondria from rat liver incorporate amino acids only into the inner membrane proteins [4–6]. However, these studies have the disadvantage of having been performed in a rather unphysiological system, in which a number of processes might be impaired, including the formation of complete polypeptide chains on and release from the mitochondrial ribosomes, combination of proteins with newly synthesized phospholipid molecules, as well as transport mechanisms. In order to avoid these difficulties and to properly evaluate the results obtained in the system *in vitro*, a study was made with intact cells. Cycloheximide, a specific inhibitor of extramitochondrial protein synthesis, can be used to differentiate between the intrinsic and extrinsic systems [7,8,25]. In the experiments reported here, the effect of this antibiotic on protein synthesis in whole *Neurospora crassa* cells was used to study biogenesis of outer and inner mitochondrial membranes.

MATERIALS AND METHODS

Cultivation of Neurospora *and Preparation of Mitochondria*

Hyphae of *Neurospora crassa* (wild type 74A) were grown under sterile conditions in Vogel's minimal medium [9] supplemented with 2% sucrose for 18 to 20 h at 25° C in 8-l bottles which were continuously aerated. The cultures were grown in a room lit with fluorescent lamps. The inoculum was such that each bottle contained 2×10^6 conidia/ml. The contents of each bottle were filtered through fine mesh gauze on a Buchner funnel and the hyphae were washed once with 1 l distilled water. The hyphae were squeezed dry and the wet weight obtained (60–90 g). They were then suspended in 10 volumes (with respect to wet weight) ice-cold isolation medium (0.44 M sucrose, 10 mM Tris buffer, pH 7.2, 2 mM EDTA) and homogenized for 30 sec in a blender. The cells were then disrupted in a grinding-mill as described by Weiss *et al.* [10]. The material emerging from the grinding mill was screened through muslin and the

cellular debris discarded. The mitochondria were isolated by fractional centrifugation of the filtrate in a Sorvall Centrifuge (rotor SS 34) at 4° C according to the following scheme: 10 min at $1500 \times g$; the resulting supernatant 30 min at $10800 \times g$; the pellets resuspended in isolation medium 10 min at $650 \times g$; the resulting supernatant 20 min at $17300 \times g$; the pellets were resuspended in isolation medium and centrifuged again 20 min at $17300 \times g$ to give a final mitochondrial pellet.

For preparation of cell fractions which sediment *at higher* centrifugal forces than mitochondria, the supernatant of the first centrifugation in which mitochondria were sedimented was centrifuged for 20 min at $27000 \times g$. The supernatant of the resulting pellet was again centrifuged for 1 h at $78500 \times g$ in a Beckman Spinco centrifuge (rotor 30). This step sedimented the cytoplasmic ribosomes as a colourless pellet with an orange layer above them which was collected by shaking in 0.1 M phosphate buffer, pH 7.2.

Preparation of Outer and Inner Membrane Fractions

Mitochondrial outer membrane was separated from inner membrane using a modified combined technique of swelling, shrinking and sonication described by Parsons *et al.* [2,11] and Sottocasa *et al.* [3] and first applied to *Neurospora* mitochondria by Cassady [12,13]. The sedimented mitochondria were suspended in 60 ml Tris-HCl 10 mM, pH 7.6, and homogenized in a Potter-Elvehjem glass homogenizer with a teflon pestle, then allowed to stand for 30 min at 0° C. Homogenisation was resumed for the last 2 min. 20 ml of shrinking solution (1.8 M sucrose, 8 mM ATP, 8 mM $MgCl_2$ neutralized with KOH to pH 7.6) were then added. Shrinking was manifested by an increase of the turbidity of the suspension. After 5 min the swollen, shrunken mitochondria were sonified in 20 ml aliquots for 3×5 sec, with cooling in an ice-bath, at setting 3 (3 A) in a Branson sonifier (Model S 75). The sonified suspensions were centrifuged for 60 min at $78000 \times g$. The combined pellets were resuspended in 5 ml of isolation medium and applied to the top of a linear sucrose density gradient containing 50 ml, and varying between 0.95 and 1.5 M sucrose in 10 mM Tris-HCl, pH 7.5. The gradient was centrifuged 1 h at $75000 \times g$ and 1° C in a Spinco ultracentrifuge, using the 25.2 rotor. The gradient was cut into 11 fractions of 5 ml each, which were then diluted to 11 ml with Tris-HCl, 10 mM, pH 7.5 and centrifuged for 1 h in the Spinco ultracentrifuge at $151000 \times g$ (rotor 50 Ti). The resulting pellets were separately resuspended in 11 ml 0.1 M potassium phosphate buffer, pH 7.2, and centrifuged for 1 h at $151000 \times g$. The resulting pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.2, to a final protein concentration of 2—5 mg/ml.

Enzyme and Cytochrome Determinations

Kynurenine hydroxylase was measured according to the method of Schott *et al.* [14], except that the test volume was reduced to 0.28 ml. The method was standardized by using 3-hydroxy-kynurenine, kindly supplied by Dr. B. Linzen. Succinate-cytochrome *c* reductase was measured according to the method of Arrigoni and Singer [15], with addition of phenazine methosulfate. Enzyme kinetics were recorded in a Beckman DK 1A spectrophotometer. Cytochrome concentrations were calculated from difference spectra recorded in a split-beam spectrophotometer designed by M. Klingenberg to measure turbid suspensions.

Protein and RNA Determinations

Protein was measured by the Biuret method [16]. For RNA determinations aliquots from each fraction were precipitated with cold $HClO_4$ (final concn. 5%), washed 4 times with cold 5% $HClO_4$ and hydrolyzed with 5% $HClO_4$ by heating for 15 min at 90° C. The absorbancy at 260 nm was read. High molecular weight RNA from yeast (Serva) was used as a standard.

Carotenoid Pigments

Carotenoid pigments were extracted according to the method of Harding *et al.* [17]. The pigments of each fraction were finally transferred to 1 ml hexane and the visible spectrum was recorded. Carotenoid content was expressed as A_{477} units/mg protein. An A_{477} units is the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 477 nm when measured in a 1 cm path length cell.

Electromicrographs

Electron micrographs were made at each step during the preparation including each of the final fractions, using a Zeiss EM-9. The samples were fixed with chromate-osmic acid and contrast made with lead citrate.

Protein Electrophoresis

Electrophoresis of the proteins of each fraction was performed on a 7.5% polyacrylamide gel, equilibrated with a medium consisting of phenol, formic acid and water (2:1:1; w/v/v) [18]. Gels were stained with amido black.

Labeling Experiments

In vitro *labeling.* Isolated mitochondria from one 8 l flask were incubated for 20 min at 32° C in the following medium [16]: 0.15 M sucrose, 30 mM KCl, 30 mM triethanolamine-HCl, 2 mM EDTA, 10 mM $MgCl_2$, 20 mM KH_2PO_4, 4 mM ATP, 0.3 mg/ml amino acid mixture (not containing leucine, iso-

leucine and phenylalanine), pH 7.6. The final volume was 60 ml. After addition of L-[^{14}C]leucine (344 mCi/mmole), L-[^{14}C]isoleucine (308 mCi/mmole) and L-[^{14}C]phenylalanine (475 mCi/mmole) (Radiochemical Centre, Amersham) 10 μCi each, the suspension was incubated in 2 ml portions with shaking. Incubation was stopped by cooling to 0° C, the suspension was then diluted by addition of 180 ml isolation medium and centrifuged for 20 min at 27000 × *g*. The resulting pellet was resuspended in 200 ml isolation medium containing 1 mM unlabeled L-leucine, L-isoleucine and L-phenylalanine and centrifuged again for 20 min at 27000 × *g*. The mitochondria were then submitted to swelling and shrinking as described above.

In vivo *labeling*. Two 8-l bottles of *Neuropora* were grown simultaneously for 18 h. To one bottle, which served as a control, L-[^{14}C]leucine, L-[^{14}C]isoleucine and L-[^{14}C]phenylalanine (specific radioactivities as in the experiments *in vitro*), 10 μCi each, were added at zero time. After 30 min a chase of 1 mM unlabeled L-leucine, L-isoleucine and L-phenylalanine was added for a further 20 min. To the other bottle cycloheximide (Sigma Chemical Co.) was added in a final concentration of 0.1 mg/ml at zero time. 5 min later, L-[^{3}H]leucine (58.2 Ci/mmole), L-[^{3}H]isoleucine (0.405 Ci/mmole) and L-[^{3}H]phenylalanine (8.3 Ci/mmole) (New England Nuclear), 0.1 mCi each, were added. After 30 min a chase of 1 mM unlabeled L-leucine, L-isoleucine and L-phenylalanine was added for a further 20 min. At the end of the chase period the contents of the two bottles were combined by filtering on the same Buchner funnel and mitochondria were prepared as described above.

Measurement of Radioactivity

Aliquots of the different fractions containing 0.2—0.3 mg of protein were applied to wet Whatman glass fibre filters and dried. The filters were then passed through 5% trichloroacetic acid (3 times), ethanol—ether 3:1, and ether. After drying the filters were counted in a Packard Tricarb scintillation counter in 10 ml toluene with PPO and POPOP.

RESULTS

Separation of Outer and Inner Membranes

When mitochondria are submitted to a swelling, shrinking, sonication procedure and then centrifuged through a linear sucrose density gradient, most of the protein is found in the lower third of the tube (fractions 6—9, Fig. 1 A), constituting a dark brown band. At the interface between the volume initially applied (fraction 1) and the top of the density gradient a red orange band can be seen. This band is collected in fraction 2. It is absent if mitochondria are applied to the gradient without previous swelling, shrinking and sonication. Fractions 1 and 2 contain 2—4% of the total protein. As shown in Fig. 1 B, kynurenine hydroxylase activity, which serves as an outer membrane marker [12, 13, 19] is concentrated in fractions 1 and 2 and is low in the other fractions. In contrast, succinate-cytochrome *c* reductase and cytochrome pigments, which serve as inner membrane markers, are concentrated in fractions 7—9 of the gradient and are low in fractions 1 and 2 (Fig. 1, C and E). From these data the contamination of outer membrane fractions by inner membrane is calculated to be in the range of 10—25%. This contamination could not be decreased by inserting a density gradient centrifugation step into the isolation procedure of the mitochondria. Fractions 6—7 show specific activities of kynurenine hydroxylase comparable with whole mitochondria. Electron micrographs show that these fractions consist mainly of whole mitochondria in which the membranes are not separated.

In order to demonstrate that the fractions at the top of the gradient consist of outer membrane and do not represent non-mitochondrial cellular constituents (extramitochondrial vesicles) enzyme activities were measured in cell fractions sedimenting at higher centrifugal forces than mitochondria. The results are given in the Table. It is seen that kynurenine hydroxylase activity is concentrated only in fraction 1 from the gradient and not in those fractions sedimenting at 27000 × *g* and 78500 × *g*. Further evidence for the mitochondrial origin of the kynurenine hydroxylase activity is afforded by the observation that this enzyme activity is absent from fraction 1 and only minimally present in fraction 2, when a mitochondrial suspension is applied to the gradient with omission of the swelling, shrinking, sonication procedure.

The sediments yielded by centrifugation of fractions 1 and 2 are red. This pigment can be readily extracted by methanol and acetone and transferred into hexane. It can be extracted into hexane from acid methanol—water but not from alkaline methanol—water. This demonstrates the acidic nature of the pigment. The carotenoid-like absorption spectrum shows a maximum at 477 nm and shoulders at 450 and 510 nm. Both extraction behaviour and the absorption spectrum strongly suggest that this pigment is neurosporaxanthin, which has been shown to be the main acidic carotenoid of *Neurospora crassa* [17, 20]. As shown in Fig. 1 D, this pigment is distributed in the various fractions of the gradient in a manner similar to kynurenine hydroxylase activity. The red pigment is concentrated only in the outer membrane fractions, and is low in those fractions sedimenting at 27000 × *g* and 78500 × *g* (Table). Therefore this pigment serves as an additional marker for outer membrane.

The distribution of RNA in the gradient expressed as μg RNA per mg protein follows a pattern very similar to succinate-cytochrome *c* reductase and the

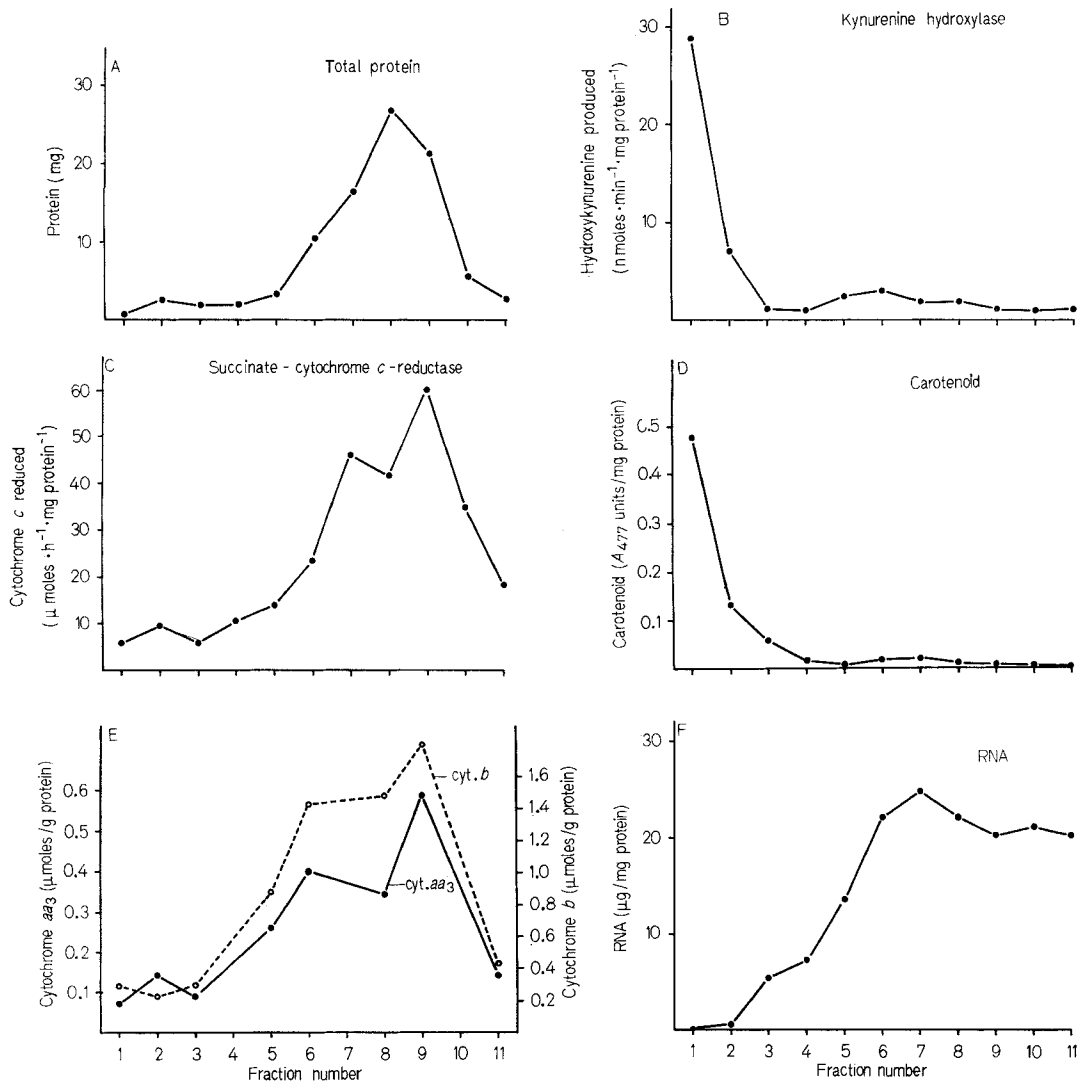
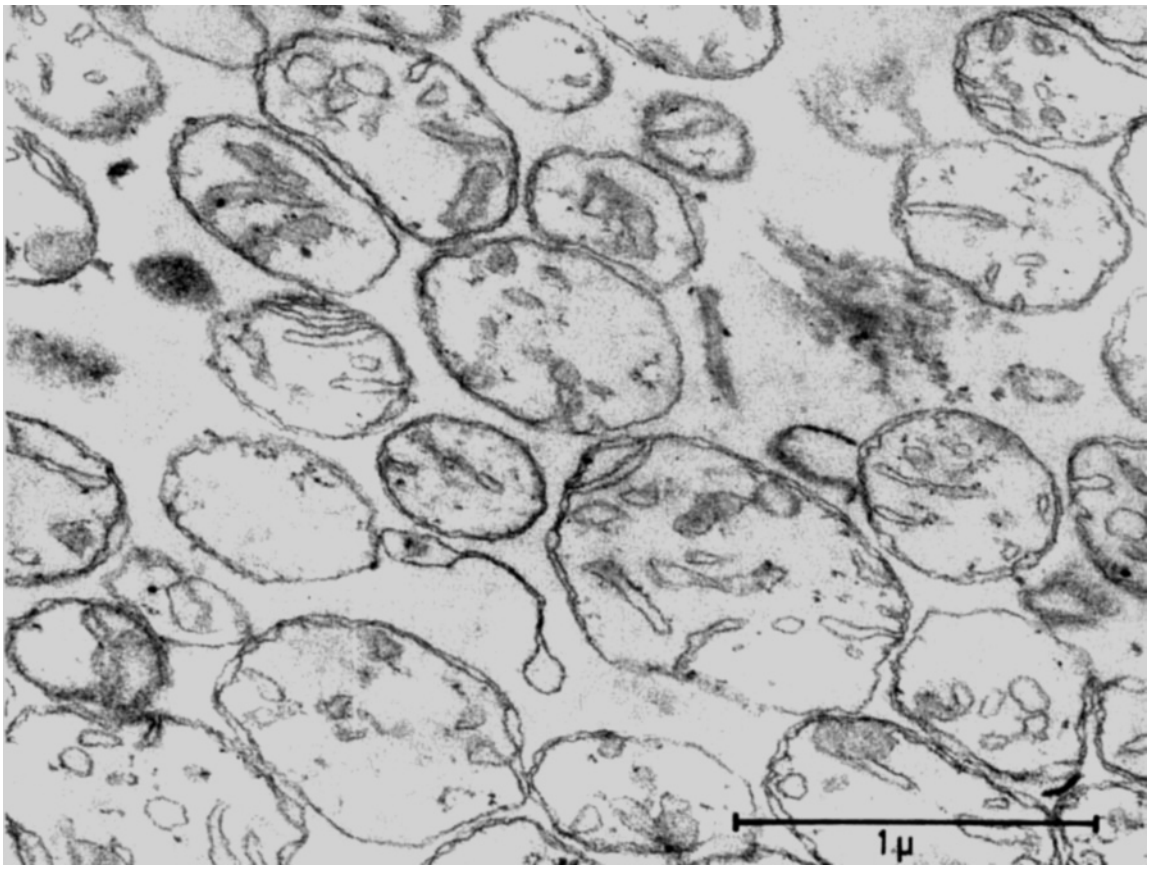


Fig. 1. *Distribution of mitochondrial components and enzyme activities in a linear sucrose density gradient through which a mitochondrial preparation was centrifuged after swelling, shrinking and sonication.* Fraction 1 represents top and fraction 11 bottom of the tube. Fraction volume is 5 ml. (A) protein; (B) kynurenine hydroxylase activity; (C) succinate-cytochrome *c* reductase activity; (D) carotenoid pigment; (E) cytochromes aa_3 (●) and *b* (○); (F) RNA

Table. *Marker enzyme activities, cytochrome and carotenoid contents of different fractions from* Neurospora *cells*
Fraction 1 (outer membrane preparation) and fraction 9 (inner membrane preparation) from gradient correspond to those from Fig. 1. Succinate-cytochrome *c* reductase activity is expressed as μmoles cytochrome *c* reduced × h^{-1} × mg $protein^{-1}$; kynurenine hydroxylase activity is expressed as nmoles hydroxykynurenine produced × min^{-1} × mg $protein^{-1}$; carotenoid pigment is expressed as A_{477} units/mg protein

Fraction	Succinate cytochrome *c* reductase	Cyto-chrome aa_3	Cytochrome *b*	Kynurenine hydroxylase	Carotenoid pigment
	μmoles × h^{-1} × mg $protein^{-1}$	μmoles/g protein		nmoles × min^{-1} × mg $protein^{-1}$	A_{477} units/mg protein
Mitochondria (extracted with 0.1 M phosphate buffer)	37.1	0.48	1.25	3.0	0.014
Fraction 1 from gradient (outer membrane)	5.8	0.07	0.30	29.4	0.459
Fraction 9 from gradient (inner membrane)	59.5	0.58	1.79	1.0	0.006
20 min 27000 × *g* pellet	10.0	—	—	0.54	0.008
60 min 78000 × *g* pellet	3.5	0.09	0.24	0.35	0.018



A

Fig. 2. *Electromicrographs taken from different steps of the swelling, shrinking and sonication procedure and from different fractions of the gradient.* (A) Whole mitochondria; (B) swollen phase; (C) shrunken phase; (D) fraction 2 from gradient (outer membrane preparation); (E) fraction 9 from gradient (inner membrane preparation)

cytochromes (Fig. 1 F). Thus, mitochondrial outer membrane prepared by this method does not contain RNA or ribosomes.

Electron micrographs were made at all steps in the procedure and of each of the eleven fractions from the sucrose density gradient. Representative samples are given in Fig. 2. Whole mitochondria with clearly identified intact inner and outer membranes and cristae are shown in Fig. 2 A. As a result of swelling, shown in Fig. 2 B, two distinct membranes can no longer be identified because of the close approximation of the two membranes in some of the swollen mitochondria and the separation and loss of outer from inner membranes. In addition, the clear profiles of the cristae are lost in the swollen mitochondria. In the shrunken phase, shown in Fig. 2 C, mitochondria, in which inner membrane is contracted away from attached outer membrane, as well as inner membranes completely devoid of outer membrane can be seen. Moreover, single membrane vesicles, which may represent detached outer membrane are also present. Fraction 2 (Fig. 2 D) as well as fraction 1 (outer membrane) consist almost entirely of single membrane vesicles of various sizes. Fraction 9 (Fig. 2 E) (inner membrane) consists of larger single membrane vesicles, retaining some profiles of cristae.

Patterns obtained by polyacrylamide gel electrophoresis are shown in Fig. 3. Fraction 1 yields essentially a single protein band, which can be identified as that corresponding to band number 11 of whole mitochondria according to Sebald *et al.* [18]. Traces of bands 4, 5, 6 and 8 can also be detected. Fraction 9 exhibits all the protein bands observed in patterns obtained by electrophoresis of whole mitochondria [18], with the notable exception, that band 11 is much weaker. The quantitative relationships for the membrane protein bands of whole mitochondria, fraction 1 and fraction 9 are seen in the densitometer tracings of the amido black stained gels (Fig. 4).

Labeling Experiments

Isolated mitochondria were incubated under conditions previously determined to be optimal with radioactive amino acids [18]. Following incubation

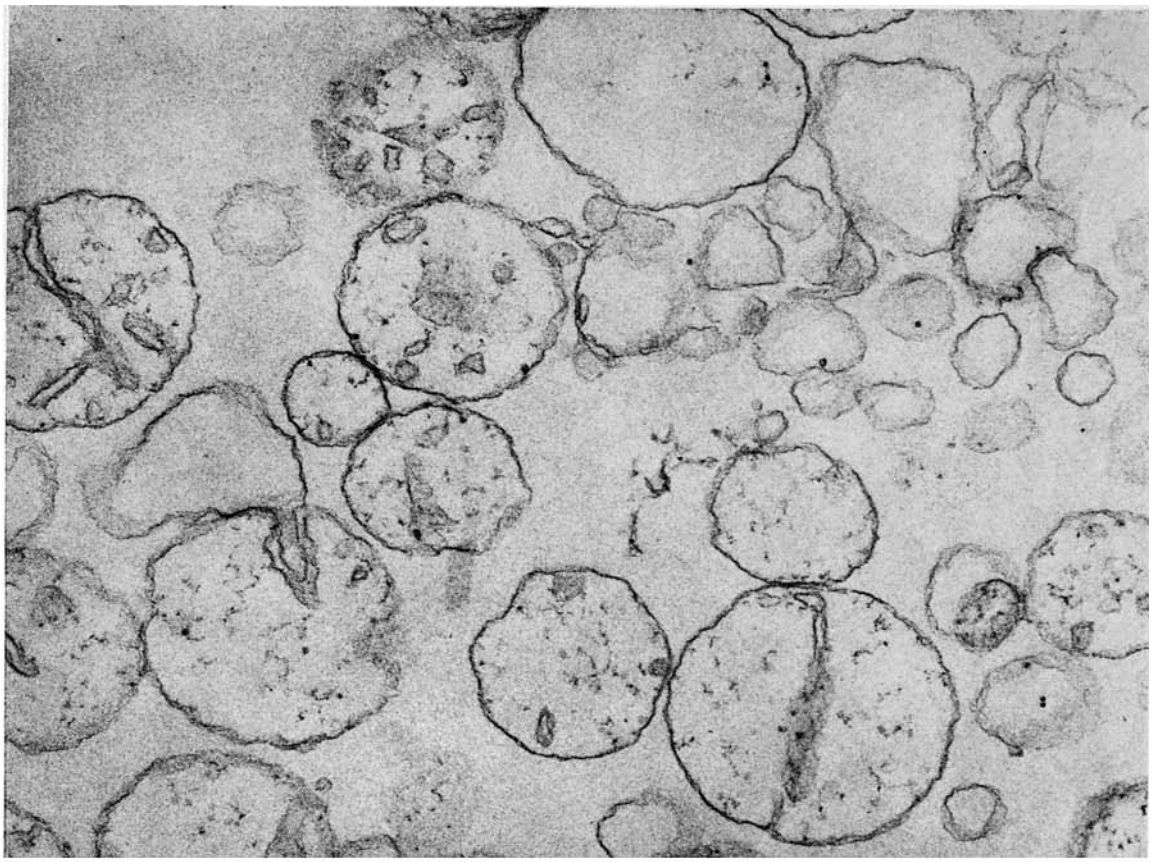


Fig. 2B

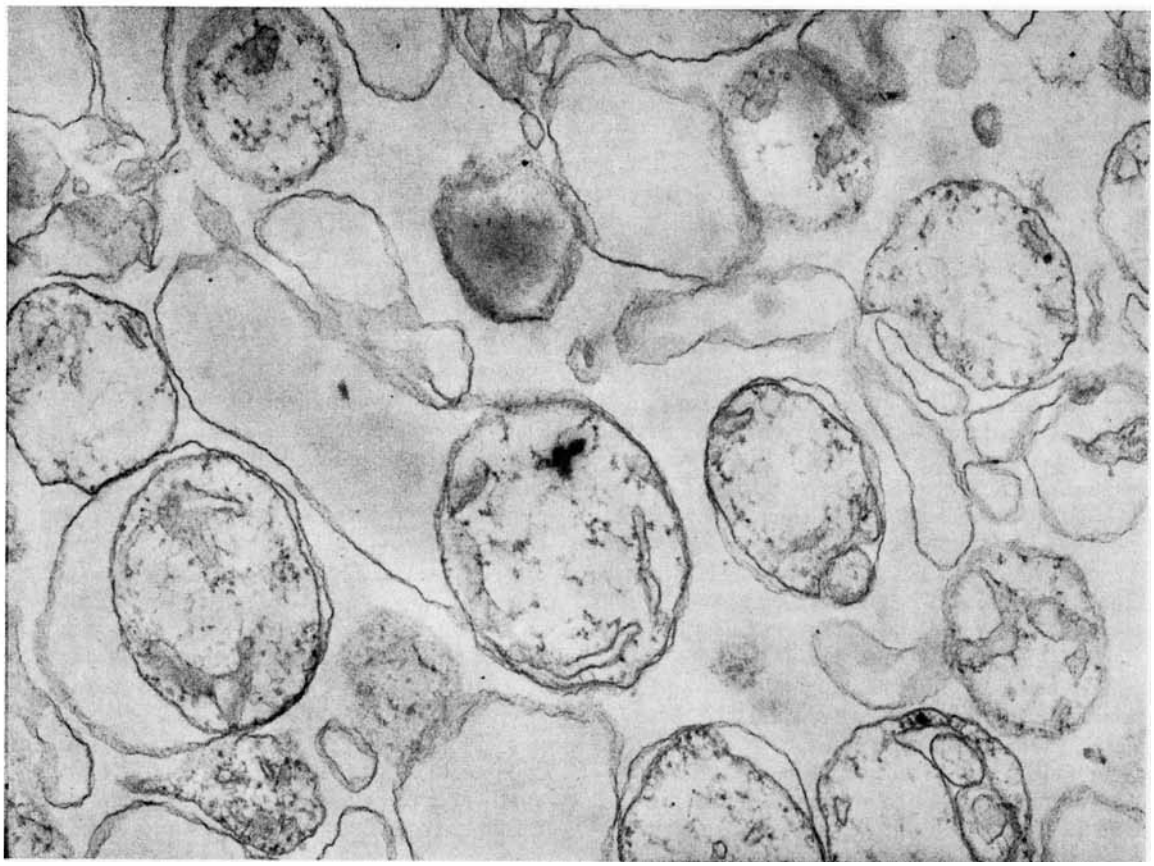


Fig. 2C

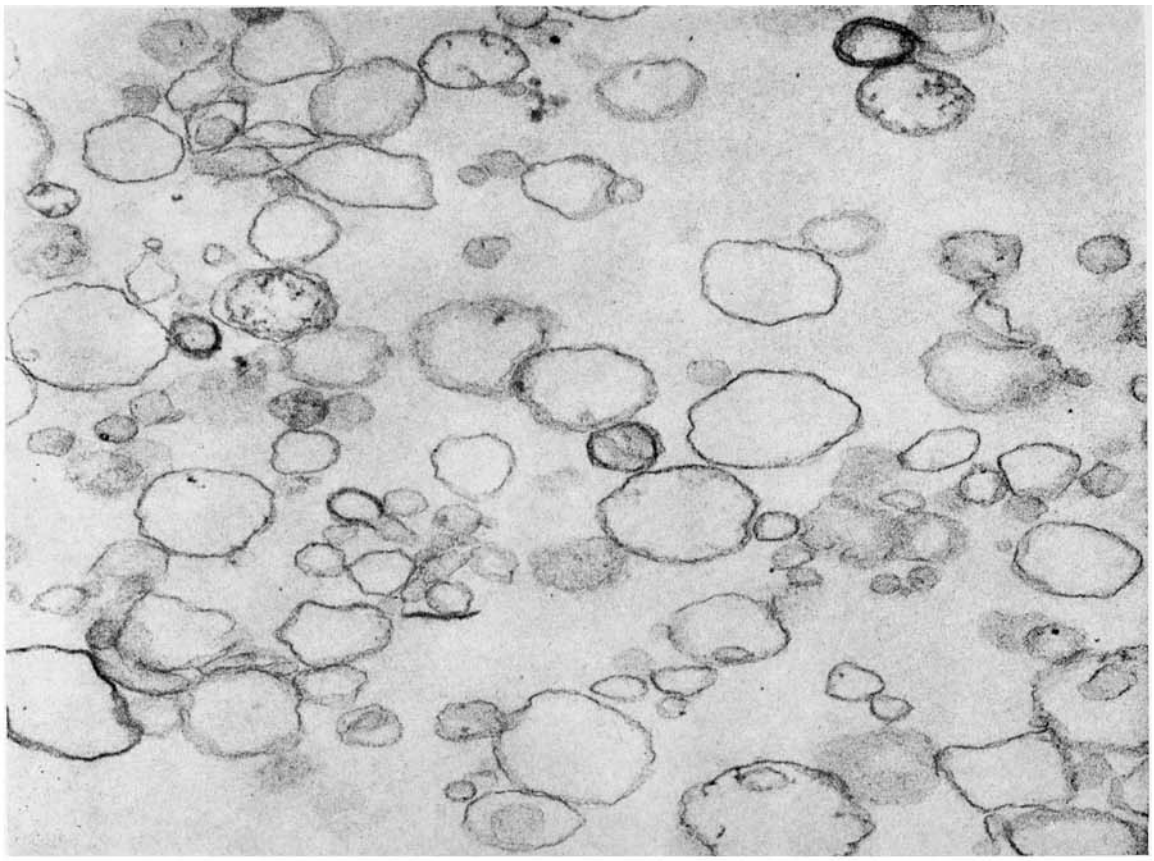


Fig. 2D

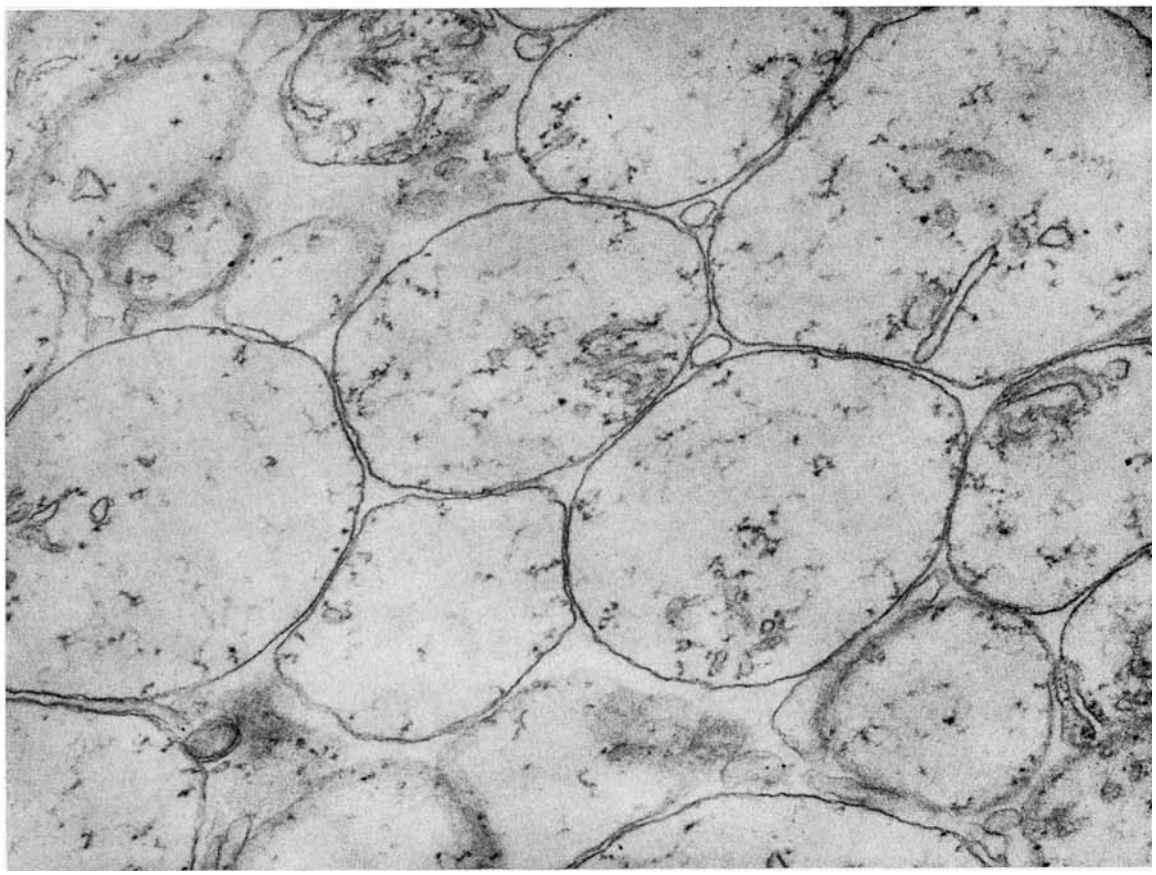


Fig. 2E

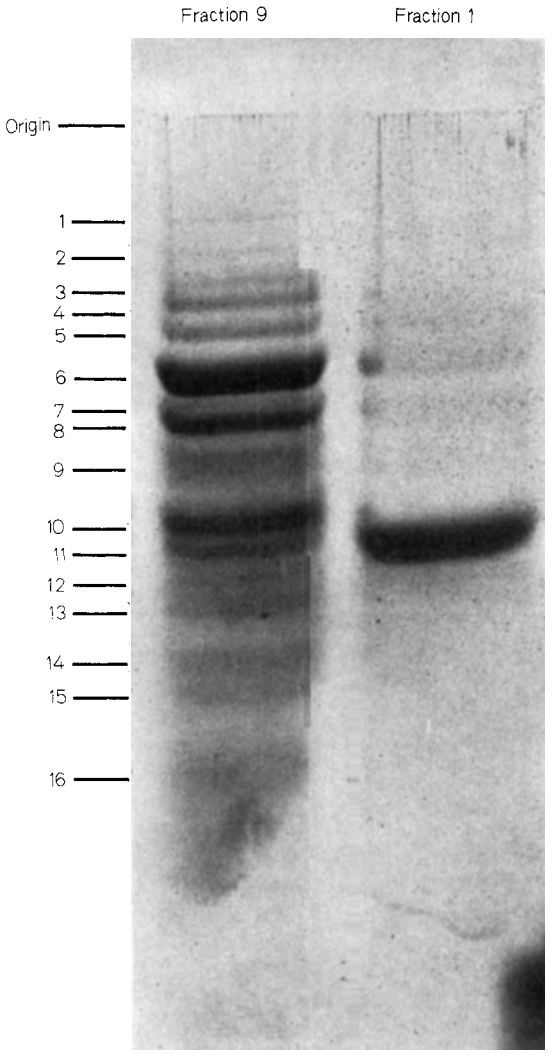


Fig. 3. *Polyacrylamide gel electrophoresis of protein from inner (fraction 9) and outer (fraction 1) membrane preparations.* Gels were stained with amido black. Bands are numbered according to Sebald *et al.* [18]

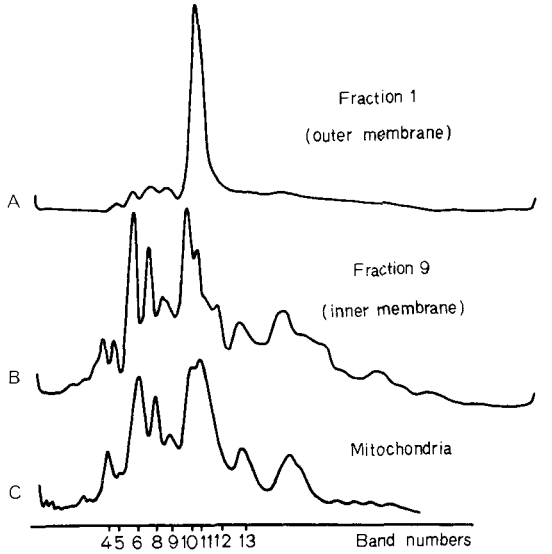


Fig. 4. *Densitometer tracings of electrophoretic patterns from proteins of outer membrane preparation (A, fraction 1), inner membrane preparation (B, fraction 9) and from unseparated mitochondrial membrane (C).* Bands are numbered according to Sebald *et al.* [18]. Absorbance of the amido black stained gels was measured at 546 nm

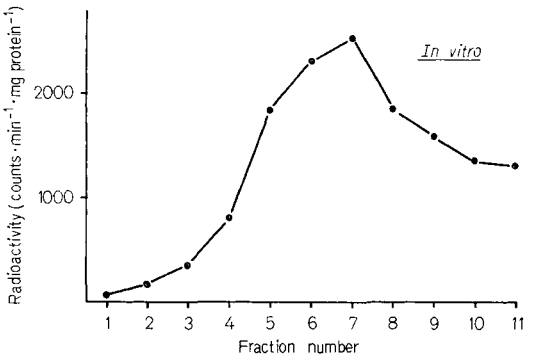


Fig. 5. *Specific radioactivities of mitochondrial outer and inner membrane fractions after incorporation of labeled amino acids into isolated mitochondria.* Fractions were obtained by swelling, shrinking and sonication and subsequent sucrose density gradient centrifugation. Fraction 1 represents top and fraction 11 bottom of the tube

the mitochondria were submitted to the swelling, shrinking, sonication procedure. The specific radioactivities of the different fractions after density gradient centrifugation are shown in Fig. 5. The specific radioactivities of fractions 1 and 2 from the top of the gradient, representing outer membrane, are low compared to those of inner membrane fractions. This indicates that polypeptide chains synthesized by mitochondrial ribosomes in isolated mitochondria are integrated into inner membrane but not into outer membrane.

In order to study the biosynthesis of outer membrane proteins in whole cells, two portions of *Neurospora* cells were incubated separately, one with ^{14}C-labeled amino acids without cycloheximide, and the other with ^{3}H-labelled amino acids in the presence of cycloheximide, as described in Methods. After labeling both portions were combined and outer and inner membranes were prepared. As shown in Fig. 6 A in the control experiment (^{14}C radioactivity) all fractions from the gradient exhibit approximately equal specific radioactivities. This indicates that in exponentially growing Neurospora cells, mitochondrial outer and inner membrane proteins are synthesized at a similar rate.

After poisoning with cycloheximide cytoplasmic protein synthesis is inhibited to 99 % [8]. The specific radioactivity corresponding to this 1 % of control was measured and subtracted from all the fractions 1 to 11. Fig. 6 B shows that in the presence of cycloheximide, inhibition of incorporation into outer membrane (^{3}H radioactivity) greatly exceeds the inhibition

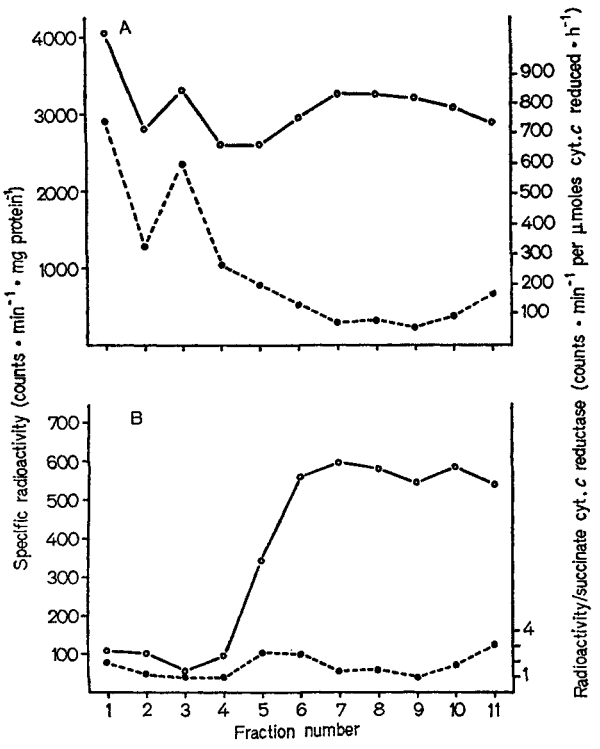


Fig. 6. *Incorporation* in vivo *of labeled amino acids into outer and inner mitochondrial membrane fractions in the presence and absence of cycloheximide.* Cells were labeled in two separate portions, one with ^{14}C-labeled-amino acids in the absence of cycloheximide, the other with ^{3}H-labeled-amino 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). ○, specific radioactivities (counts × min^{-1} × mg protein^{-1}); ●, ratio radioactivity/succinate-cytochrome *c* reductase activity (counts × min^{-1} per μmoles cytochrome *c* reduced × h^{-1})

of incorporation into inner membrane fractions. Furthermore, specific radioactivity and succinate-cytochrome *c* reductase activity have a very similar distribution in the gradient (with exception of fractions 10 and 11). This is reflected in the ratio radioactivity/succinate-cytochrome *c* reductase activity also shown in Fig. 6B. It is very similar in fractions 1 to 2 and 8—9. This indicates that radioactivity present in the outer membrane fractions is due to contamination by inner membrane. In agreement with this observation the ratio radioactivity/succinate-cytochrome *c* reductase activity of the same fractions is very different in the control experiment (Fig. 6A). These observations demonstrate that in the presence of cycloheximide the labeling of outer membrane protein is less than 2—3% of that of the inner membrane.

DISCUSSION

The results obtained by separating outer and inner membranes of *Neurospora crassa* mitochondria reported here confirm the work of Cassady [12,13], who has shown that the combined swelling, shrinking, sonication procedure developed by Parsons *et al.* [2, 11] and Sottocasa *et al.* [3] for liver mitochondria, can also be applied to *Neurospora* mitochondria. Electronmicrographs taken from the subsequent steps of this procedure demonstrate a very similar response of both types of mitochondria to swelling and shrinking. Furthermore, the electronmicrographs disclose the separation on the sucrose density gradient into lighter single membrane vesicles on top of the gradient, corresponding to outer membrane, and heavier single membrane vesicles, near the bottom of the gradient, with the characteristics of inner membrane. However, the separation appears not to be a complete one, since between these outer and inner membrane fractions double membrane vesicles are found.

Further identification of these vesicles is established by marker enzyme activities, using succinate-cytochrome *c* reductase and cytochromes aa_3 and *b* as inner membrane markers [2,3,21]. *Neurospora* mitochondria lack rotenone-insensitive NADH-cytochrome *c* reductase and monoamine oxidase activities [13], which are present in liver mitochondria [3,21]. Therefore, kynurenine hydroxylase, first shown by Cassady to be localized in outer mitochondrial membrane of *Neurospora* [12] was used as a marker enzyme.

A red carotenoid pigment, tentatively identified as neurosporaxanthin [20] was readily extracted from outer membrane fractions. Since it was present only in very low concentrations in inner membrane fractions, or in the extramitochondrial fractions requiring higher centrifugal forces for sedimentation, it was found to serve as a good marker for outer membrane. The function of this carotenoid is unknown. It may be speculated that it serves as a free radical scavanger to protect the phospholipids of the outer membrane from oxidation induced by light and/or to absorb light and thus prevent damage to the electron transfer system in the inner mitochondrial membrane [22]. As shown by Harding *et al.* [17] the formation of the carotenoid pigments in *Neurospora* is dependent on light. *Neurospora* hyphae grown in the dark show very low carotenoid concentrations.

In contrast to the outer mitochondrial membrane of rat liver [23] that of *Neurospora* shows essentially one single protein band on polyacrylamide gel electrophoresis. The minor bands present can be considered to be contaminating inner membrane protein. This is substantiated by the observation that these bands correspond to major bands of the inner membrane. Furthermore, the contamination of the outer membrane preparations by inner membrane, as estimated by marker enzyme activities, corresponds

to the proportions of these minor bands as evaluated by absorbance of the gels. These findings suggest that more than 95% of the protein component of the outer membrane of *Neurospora* consists either of one single protein or of a greater number of proteins with identical electrophoretic mobilities or with the ability to form an aggregate under the conditions of the electrophoresis.

Another important difference between *Neurospora* and the rat liver system is the absence of microsomal contamination of outer membrane fractions, since no membrane system equivalent to liver endoplasmic reticulum is present in *Neurospora*. This is also reflected in the observation that RNA and therefore ribosomes are not detected in the outer mitochondrial membrane in contrast to outer membrane preparations from rat liver, in which even higher RNA concentrations than in inner membrane were found [5].

The results of incorporation of labeled amino acids into isolated mitochondria reported here are in agreement with those obtained with rat liver mitochondria [4—6]. They suggest that the outer membrane proteins cannot be synthesized in the absence of the cytoplasmic protein-synthesizing system. This is confirmed and extended by studies with whole cells, to which cycloheximide was applied to block cytoplasmic protein synthesis and leave mitochondrial amino acid incorporation unimpaired. They indicate that at least 97—98% of outer membrane protein of mitochondria must come from the cytoplasmic system.

This conclusion is strengthened by the results of electrophoresis of the proteins from the outer and inner membrane fractions. The band which constitutes almost entirely the protein of outer membrane is not labeled in experiments in which only mitochondrial ribosomes are synthesizing polypeptide chains, as demonstrated either in isolated mitochondria, or in whole cells, in which the cytoplasmic ribosomes are poisoned with cycloheximide. This has been shown in previous work from this laboratory by Sebald *et al.* [8,18]. Of added interest are experiments with the *mi-1* (poky) mutant of *Neurospora* [24]. When outer and inner mitochondrial membranes of this mutant are separated by the techniques described here and the protein fractions subsequently subjected to polyacrylamide gel electrophoresis, the same band as in wild type appears in the outer membrane fraction. This shows that the gross composition of outer membrane is unchanged in this mutant, in which the gross composition of inner membrane is known to be altered [18].

These studies provide conclusive evidence that the extramitochondrial cytoplasmic protein synthesizing system is responsible for more than 97 to 98% of the outer membrane protein and that the intrinsic system of mitochondria contributes only to the biogenesis of inner membrane proteins.

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