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MEMBRANE SEPARATION AND BIOGENESIS OF THE OUTER MEMBRANE OF YEAST MITOCHONDRIA

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SUMMARY

1. Isotonic swelling and shrinking of the mitochondria of *Saccharomyces cerevisiae* in the presence of valinomycin and subsequent sucrose gradient centrifugation resulted in the separation of the inner and outer mitochondrial membranes.

Succinate: cytochrome *c* reductase and malate dehydrogenase served as markers for the inner membrane and the matrix, respectively, adenylate kinase for the intermembrane space.

2. The outer membrane fraction is characterized by the activities of both antimycin A-insensitive NADH: cytochrome *c* reductase and kynurenine hydroxylase. The latter is shown to be exclusively bound to the outer mitochondrial membrane in yeast. Kynurenine hydroxylase exhibits a sharp pH optimum at about pH 7.4. Yeast mitochondria fail to show monoamine oxidase activity.

3. The products of mitochondrial and cytoplasmic protein synthesis in *Saccharomyces cerevisiae* were each selectively labelled *in vivo*. The fractions obtained after membrane separation were characterized by their ^{3}H to ^{14}C ratio. The outer membrane of yeast mitochondria is not pulse-labelled by radioactive leucine incorporation in the presence of cycloheximide and hence is apparently not synthesized by the mitochondrial protein synthesizing system.

More than one third of the inner membrane protein is synthesized on mitochondrial ribosomes.

4. As estimated from enrichment in enzyme activity and in specific radioactivity, the outer membrane fraction contains 6–8 %, the inner about 30 % of the total protein in yeast mitochondria.

5. Intact mitochondria, outer and inner membranes exhibit densities of 1.173 g/cm^3, 1.084 g/cm^3 and 1.190 g/cm^3, respectively.

6. All mitochondrial subfractions obtained after membrane separation were examined for their morphological appearance by electron microscopy.

INTRODUCTION

Mitochondrial membrane separation was first achieved by Parsons *et al.*[1], Sottocasa *et al.*[2] and by Schnaitman *et al.*[3] with mammalian liver mitochondria. The work of these authors contributed fundamentally to the view that the enzymes of the respiratory chain are located in the inner membrane. Greenawalt and Schnaitman[4]

have recently demonstrated that mitochondrial malate dehydrogenase, isocitrate dehydrogenase and glutamate dehydrogenase are matrix enzymes in mammals. Our results confirm these data for yeast mitochondria. Since yeast mitochondria apparently do not contain monoamine oxidase activity, both antimycin A-insensitive NADH:cytochrome *c* reductase (EC 1.6.99.3) and kynurenine hydroxylase (EC 1.14.1.2) were used for the identification of the outer mitochondrial membrane (*cf.* Greenawalt and Schnaitman[4], Okamoto *et al.*[5]).

In eucaryotic microorganisms such as yeasts, the products of mitochondrial protein synthesis can be specifically labelled in the presence of cycloheximide, which is known to be a powerful inhibitor of ribosomal function in the cytoplasm. Mitochondrial protein synthesis is unaffected[6–9]. Erythromycin and chloramphenicol on the other hand are known to block the residual synthesis of mitochondrial proteins, not inhibited by cycloheximide[10–12]. This allows an almost selective labelling of all proteins of cytoplasmic ribosomal origin. Thus the separation of outer and inner membranes of mitochondria is a useful tool in elucidating biogenesis and function of the two membrane systems. Membrane separation will provide evidence whether the outer membrane of yeast mitochondria is synthesized entirely by cytoplasmic ribosomes or whether some of its proteins originate from the mitochondrial protein synthesizing system. With rat liver mitochondria labelled *in vitro*, Neupert *et al.*[13] found that almost no radioactivity was incorporated into the outer membrane. Recently strong evidence for the nonmitochondrial origin of the proteins of the outer mitochondrial membrane has also been presented for *Neurospora crassa*[14]. This means that the proteins contributing to the outer membrane of mitochondria are exclusively synthesized by cytoplasmic ribosomes. The results presented here confirm this view for yeast mitochondria.

MATERIALS AND METHODS

Chemicals

The chemicals used were of the highest grade of purity available. Cycloheximide was obtained from Serva, Heidelberg, chloramphenicol from Bayer, Leverkusen, and erythromycin glucoheptonate from Schering AG, Berlin. The following radiochemicals were obtained from the Radiochemical Centre Amersham: L-[^{3}H]leucine (22 Ci/mmole), L-[^{3}H]isoleucine (15 Ci/mmole), L-[^{3}H]valine (18 Ci/mmole), L-[^{14}C]leucine (344 mCi/mmole), L-[^{14}C]isoleucine (312 mCi/mmole) and L-[^{14}C]valine (260 mCi/mmole).

Cell culture and preparation of mitochondria

Cells of the haploid yeast strains *Saccharomyces cerevisiae* A 1327 A *a*, ad_2, leu_1, and M 12 *a*, ura_3, ilv_5, try_2 were grown as described previously[15]. Derepressed cells were grown in 2 % galactose as carbon source and harvested at a cell titer of $2–4 \cdot 10^7$ cells$\cdot$ml^{-1}. Conversion of the cells to spheroplasts was performed according to Ohnishi and Hagihara[16] as modified by von Jagow and Klingenberg[17]. Mitochondria were purified by differential centrifugation of the cell lysate at 4 °C: 1500 × *g* for 15 min and the resulting supernatant at 7500 × *g* for 30 min. Crude mitochondria were layered on top of a linear sucrose gradient composed of 68–41 % sucrose (w/v) buffered with 20 mM Tris–HCl, pH 7.4, 1 mM EDTA. The tubes were centrifuged in a Spinco SW 27 rotor at 27000 rev./min for 90 min at 4 °C.

Microsomes were prepared from the 7500 × *g* supernatant of the crude mitochondrial sediment. A pellet obtained by centrifugation at 15000 × *g* for 30 min was discarded and the microsomal fraction collected after sedimentation for 60 min at 105000 × *g*.

Isotonic swelling and membrane separation

Isotonic swelling of the mitochondria was performed in a medium containing 0.14 M sucrose, 5 mM KCl, 20 mM Tris–H_2SO_4, pH 7.4, at 0 °C. The protein concentration was adjusted to 10–30 mg/ml. 100 μg valinomycin in 100 μl ethanol was added per g of protein. After 5 min, 3 vol. of ice-cold shrinking buffer containing 1.8 M sucrose, 4 mM ATP, 4 mM $MgSO_4$ and 0.1 % bovine serum albumin were added under gentle homogenization in a teflon in glass homogenizer. After 5 min more, the mixture was diluted to a final concentration of 36 % sucrose (w/v).

All subsequent operations were performed at 4 °C. The membrane fractions were first separated by sequential centrifugation at 6000 × *g* for 10 min, 15000 × *g* for 15 min and 65000 × *g* for 30 min. The latter pellet was discarded. The resulting supernatant was diluted 1:1 with a buffer containing 0.25 M mannitol, 20 mM Tris, 1 mM EDTA, pH 7.4, and 0.05 % bovine serum albumin and recentrifuged at 105000 × *g* for 60 min. The resulting supernatant was called the soluble fraction. The pellet contained outer membranes.

The 15000 × *g* and 105000 × *g* pellets were homogenized and layered on top of linear gradients of 68–38 % and 44–17 % (w/v) buffered sucrose, respectively. The fractions were centrifuged at 27000 rev./min for 135 min at 4 °C in a SW 27 rotor in a Spinco ultracentrifuge. The tubes were punctured at the bottom and the contents pumped off. Elution diagrams were registered by recording the absorbance at 420 nm in a split beam spectrophotometer. A micro flow cell of 10 mm path length was used. Fractions of 20 drops were collected. Densities of the fractions were determined pyknometrically.

Enzyme assays

Assays were performed in cuvettes of 1 ml volume and 10 mm pathlength, if not otherwise stated. Cytochrome oxidase activity is calculated from the first-order velocity constant according to Smith and Conrad[18]. The activity of malate dehydrogenase is given as $\mu moles \cdot mg^{-1} \cdot min^{-1}$, of kynurenine hydroxylase in $pmoles \cdot mg^{-1}$, of all the other enzymes as $nmoles \cdot mg^{-1} \cdot min^{-1}$.

Malate dehydrogenase (EC 1.1.1.37) was determined according to Ochoa[19] and succinate: cytochrome *c* reductase (EC 1.3.99.1) according to Arrigoni *et al.*[20]. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed as described by Yonetani[21] and adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3) as described by Schnaitman and Greenawalt[3]. Microsomal NADPH: cytochrome *c* reductase was recorded according to Masters *et al.*[22].

The absorption coefficients used were $\varepsilon_{550} = 18.5 \cdot 10^6$ $mole^{-1} \cdot cm^2$ for cytochrome *c* reduction and $\varepsilon_{340} = 6.22 \cdot 10^6$ $mole^{-1} \cdot cm^2$ for the reduction of NAD^+ and $NADP^+$.

Kynurenine hydroxylase (EC 1.14.1.2) was assayed in the following manner: Both cells contained in a volume of 1.4 ml: 0.2 mmole of Tris–acetate, pH 7.4, 40 μmoles of KCl, 0.9 μmoles of KCN (pH 7.4), and 0.4 μmoles of NADPH. The enzyme

suspension was taken from the sucrose gradients and added to both cells (0.2–1 mg of protein in a volume of 0.5 ml each). After the determination of the blank reaction, the substrate specific reaction was started by the addition of 0.1 ml of 1 mM L-kynurenine sulfate in water. The reference received 0.1 ml of water instead. The decrease in absorbance at 340 nm was recorded over a period of at least 20 min for both the blank reaction and for the substrate-dependent reaction. An adsorption coefficient $\varepsilon_{340} = 7.3 \cdot 10^6$ mole$^{-1} \cdot$cm^2 was used, which accounts for the contribution of 3-hydroxykynurenine.

The assay mixture of antimycin A-insensitive NADH:cytochrome *c* reductase (EC 1.6.99.3) contained in 1.8 ml: 1.3 mmoles of mannitol, 40 μmoles of Tris–maleate, 10 μmoles of potassium phosphate, 20 μmoles of KCl, 2 μmoles of EDTA, 0.9 μg of antimycin A at a final pH of 6.5. After the addition of 0.1 ml enzyme solution, the reaction was started by adding 0.1 ml of 2 mM NADH in water. The results were corrected for blank reaction.

Protein was determined according to the method of Lowry *et al.*[23] with bovine serum albumin as standard.

Labelling experiments in vivo

Cells were washed three times at 30 °C with sterile Wickerham synthetic medium[24] containing half of the initial hexose concentration, resuspended in the same medium and aerated for 15 min at 30 °C. The cell titer was $5–8 \cdot 10^8$ per ml. Cycloheximide (200 μg/ml) was given 3 min[9] and both erythromycin (2 mg/ml) and chloramphenicol (4 mg/ml) 15 min prior to the addition of the label. In double labelling experiments the cell material was divided into two aliquots. One of them was preincubated with cycloheximide and labelled with 20 μCi of [^{14}C]leucine or with 10 μCi each of [^{14}C]isoleucine and [^{14}C]valine depending on the strain used. The other was preincubated with erythromycin and/or chloramphenicol and labelled with 50 μCi of [^{3}H]leucine or 25 μCi each of [^{3}H]isoleucine and [^{3}H]valine, respectively. After 20 min the incubation mixtures were chased by a 10000-fold molar excess of unlabelled leucine or isoleucine and valine, respectively. After another 20 min both cultures were poured on ice, mixed and washed twice in a buffer containing 0.25 M mannitol, 0.02 M Tris–HCl, 1 mM EDTA buffer, pH 7.4, and 1 mM in the respective unlabelled amino acids. Controls for bacterial contamination were performed by plating 0.1 ml of the incubation mixture on Wickerham agar containing 10 μg/ml cycloheximide.

Preparation of submitochondrial particles

Mitochondria or mitochondrial subfractions were suspended in 0.1 M phosphate buffer, pH 7.4, 1 mM EDTA and sonicated for 2 min (Branson B 12 sonifier, maximum power, pulses of 10 s with intervals of 1 min during which the mixture was cooled with ice). The suspension was then centrifuged at 105000 × *g* for 60 min and the resulting sediment resuspended in the same medium. The sonication was repeated twice with subsequent centrifugations at 155000 × *g* for 30 min.

Preparation of samples and liquid scintillation counting

For liquid scintillation counting, protein was precipitated by 6% trichloroacetic acid and washed as described by Simkin and Work[25]. The samples were dis-

solved in 0.3 ml Soluene (Packard Instruments). The scintillation cocktail consisted of 10 ml toluene containing 5 g/l PPO and 0.3 g/l dimethyl-POPOP. The vials were counted in a Packard liquid scintillation counter Model 3003. The efficiency for ^{3}H was 20.5 % and for ^{14}C 56.5 %.

Electron micrographs

Thin sections: After fixation with chromate–osmic acid the samples were contrasted in a neutralized solution of 1 % phosphotungstic acid and 0.5 % uranyl acetate in 70 % aqueous acetone for 60 min. After dehydration the pellet was embedded in Vestopal and thin-sectioned with glass knives. The contrast was enhanced facultatively with 0.1 % phosphotungstic acid in water. The thin sections were mounted on Formvar coated copper grids and viewed in a Zeiss EM9A electron microscope at a magnification of 18000 diameters. Spreading and negative staining: After prefixation with 1 % osmic acid at 0 °C for 30 min, mitochondrial samples were withdrawn by a pin point and spread on the surface of a drop of neutralized 3 % phosphotungstic acid solution containing 0.01 % bovine serum albumin, dropped on a sheet of dental wax. The specimens were picked up by Formvar coated copper grids. Excess stain was removed by filter paper. The sample was then air-dried and viewed as described above.

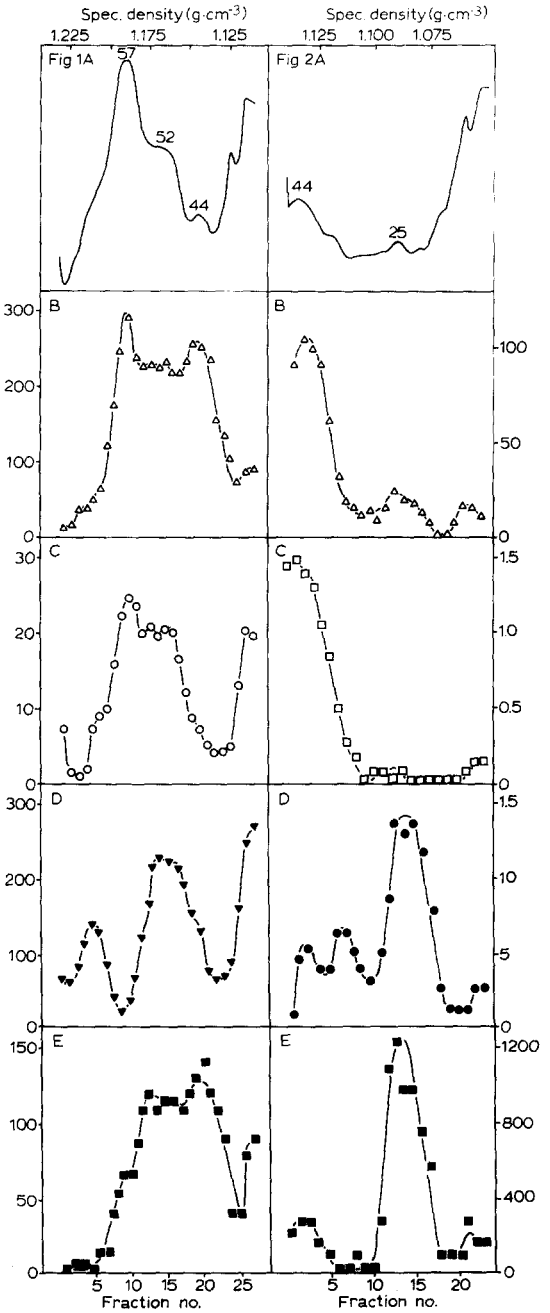
RESULTS

Membrane separation

Mitochondrial membrane separation has hitherto been achieved by either hypotonic swelling[1,2] or mild detergent treatment[3], sometimes combined w th gentle sonication. With mitochondria of *Saccharomyces cerevisiae* these standard methods lead to extensive structural damage as shown by sucrose gradient centrifugation and subsequent electron microscopy of the various fractions. Inner and outer membranes can hardly be detected and characterized by means of their specific enzymatic properties. Apparently, the outer membrane is quite stable while the inner membrane is extremely sensitive to hypotonic treatment and mechanical shearing and/or sonication. In this study mitochondria used for membrane separation were checked for their integrity prior to the operations.

An excessive swelling of the mitochondria by uptake of K^{+} in the presence of valinomycin in an isotonic medium eliminated these difficulties. Shearing by homogenization in a Potter–Elvehjem homogenizer was minimized during the swelling period. The outer envelopes were torn off the inner membranes by successive shrinking and gentle shearing. The amount of shrinking was checked by measuring the increase in turbidity at 630 nm.

The three pellets obtained by differential centrifugation at 6000 $\times$ *g*, 15000 $\times$ *g*, and 105000 $\times$ *g*, respectively, (see Materials and Methods) were further purified by three different sucrose gradient centrifugations. The 6000 $\times$ *g* pellet contains mainly intact mitochondria. Figs 1 and 2 show the elution diagrams and the specific activities of some marker enzymes in the gradients of the 15000 $\times$ *g* and 105000 $\times$ *g* pellets mentioned above. It can be seen from Fig. 1 that fraction 57 banding at a density of 1.190 $g \cdot cm^{-3}$ (corresponding to 57 % sucrose) is the main product of this isotonic membrane separation. In this peak, succinate:cytochrome *c* reductase, cytochrome *c*



Figs 1 and 2. Elution diagrams of sucrose gradient tubes. Matrix or inner membrane enzymes (open symbols), enzymes of the outer membrane or intermembrane space (solid symbols). The fraction numbers above the various peaks of the spectrophotometric traces (A) correspond to the percentage of sucrose, at which the fractions band.

Fig. 1. Elution diagram of the 15000 × *g* pellet. (A) Absorbance at 420 nm. (B) Succinate:cytochrome *c* reductase. (C) Malate dehydrogenase. (D) Adenylate kinase. (E) Kynurenine hydroxylase.

Fig. 2. Elution diagram for the 105000 × *g* pellet. (A) Absorbance at 420 nm. (B) Succinate:cytochrome *c* reductase. (C) Cytochrome oxidase. (D) Antimycin A-insensitive NADH:cytochrome *c* reductase. (E) Kynurenine hydroxylase.

oxidase and malate dehydrogenase (*cf.* Table I) are enriched by about 35 % on the average. (Table I represents the results of an experiment different from that shown in Figs 1 and 2.) Antimycin A-insensitive NADH:cytochrome *c* reductase, kynurenine hydroxylase and adenylate kinase are absent from this fraction. The ratio of succinate: cytochrome *c* reductase to kynurenine hydroxylase reaches a sharp maximum in this peak (not shown in the graph). From these data it is concluded that this fraction contains inner membranes.

Intact mitochondria band at a density of 1.173 $g \cdot cm^{-3}$ (Fraction 52) as demonstrated by the elution diagram of freshly prepared mitochondria. The rather low peak of a fraction banding at a density of 1.144 $g \cdot cm^{-3}$ (44 % sucrose) is the same as found with ruptured mitochondria. It exhibits the same enzyme activities as intact mitochondria except matrix enzymes and adenylate kinase. In pure preparations of this fraction, the membrane-bound enzymes are enriched as compared to intact mitochondria (*cf.* Table I). The fraction found with 25 % sucrose banding at a density of 1.084 $g \cdot cm^{-3}$ (*cf.* Fig. 2) contains outer membranes as indicated by the steep increase in kynurenine hydroxylase and antimycin A-insensitive NADH:cytochrome *c* reductase activities. The ratio of kynurenine hydroxylase to succinate: cytochrome *c* reductase gives a sharp maximum for this peak (not shown in the graph).

Enzyme activities

In contrast to mammalian mitochondria no monoamine oxidase activity could be detected in the outer membrane fraction of the yeast organelle. The assay method of Tabor *et al.*[26] and the much more sensitive test with *p*-dimethylaminobenzylamine as a substrate have been tried. A small activity, however, found in intact yeast mitochondria is enriched with the inner membrane fraction and is ascribed to the unspecific action of some other oxidase located in the inner membrane.

Okamoto *et al.*[5] showed kynurenine hydroxylase to be exclusively bound to the outer membrane of mammalian mitochondria. Since there is no evidence that this applies also to yeast mitochondria, kynurenine hydroxylase together with antimycin A-insensitive NADH:cytochrome *c* reductase were used as markers for the outer membrane. Though the latter enzyme is agreed to be contained in this fraction[4], it is not a specific marker, since microsomes also show antimycin A-insensitive NADH: cytochrome *c* reductase activity (*cf.* Table I). The coincidence of both peaks of activity, however, demonstrates that kynurenine hydroxylase is an enzyme of the outer mitochondrial membrane in yeast as well.

Kynurenine hydroxylase activity of intact yeast mitochondria is extremely low. In four buffer systems it showed a rather sharp pH optimum at about pH 7.4. Tris–acetate buffer, containing 20 mM KCl was found to be most suitable. In an assay mixture including 0.5 % of a nonionic detergent such as Tween 80, Brij 35 or Lubrol WX, the activity was lost. Addition of FAD or FMN led to no further activation. The enzyme can be stored at −18 °C for at least 3 weeks without detectable loss of activity. Table I shows that respiratory enzymes and malate dehydrogenase are enriched in the inner membrane fraction while kynurenine hydroxylase and adenylate kinase are missing.

According to Table I the inner membrane fraction (No. 57) is contaminated by whole mitochondria at about 10 %, as estimated on the basis of the residual marker enzyme activity of the outer membrane. Outer membranes contain 8 % of the suc-

TABLE I

DISTRIBUTION OF SPECIFIC MARKER ENZYMES IN MITOCHONDRIAL SUBFRACTIONS AFTER MEMBRANE SEPARATION

Experiment is different from that in Figs 1 and 2. Control mitochondria have not been purified by gradient centrifugation. The fraction numbers (57, 52, 44 and 25) correspond to the percentage of sucrose, given by the banding density in a sucrose density gradient. All the enzyme activities except NADH:cytochrome *c* reductase were measured after freezing and thawing of the mitochondrial suspensions. The activities are given as substrate conversion per minute per mg of protein as described under Materials and Methods.

Enzymes tested	*Marker enzyme specific for*	*Intact mitochondria (control)*	*Mitochondrial fractions after membrane separation*				*Soluble fraction*	*Microsomes*
			57	*52*	*44*	*25*		
Cytochrome *c* oxidase	Inner membrane	2.38	3.26	2.34	4.61	0.0	0.04	0.08
Succinate:cytochrome *c* reductase		270	455	330	585	27	65	2
Malate dehydrogenase		28.5	37.8	29.1	8.0	0.8	52.1	0.2
Glutamate dehydrogenase	Soluble matrix	63	89	69	0.5	0.0	—	—
Isocitrate dehydrogenase (NAD)		164	240	190	0.5	0.0	430	—
Adenylate kinase	Intermembrane space	225	45	260	65	22	720	195
Antimycin A-insensitive NADH:cytochrome *c* reductase	Outer membrane	2.7	0.3	2.8	5.9	20.5	1.3	8.7
Kynurenine hydroxylase		105	12	130	277	1510	10	0.0
NADPH:cytochrome *c* reductase	Microsomes	6.8	0.0	3.0	4.1	5.3	0.4	36.4

cinate:cytochrome *c* reductase activity of whole mitochondria (or 6 % of the inner membranes) and 10–15 % of microsomal contamination. (The latter may be calculated from the NADPH:cytochrome *c* reductase activity of Fraction 25 and of purified microsomes).

After sonication of both intact mitochondria and the inner membrane fraction and subsequent centrifugation and washing of the sediment, malate dehydrogenase, isocitrate dehydrogenase (NADP) and glutamate dehydrogenase (NAD) (and some NADH:cytochrome *c* reductase) are found in the supernatant. These enzymes are thus demonstrated to be of matrix origin.

Labelling experiments

Labelling experiments were performed with yeast strains defective in leucine or isoleucine–valine synthesis in order to achieve a constant rate of radioactive precursor incorporation into the cell material (*cf.* ref. 9). Dilution of labelled amino acids after transport into the cell by the cellular pool was thus kept at a minimum. The concentrations and the preincubation times for cycloheximide and erythromycin applied in these experiments have been found to be optimal for achieving utmost inhibition of the respective protein synthesizing system without influencing the other one[9,12]. In the presence of 200 μg cycloheximide per ml incubation medium, the radioactivity incorporated into the 105000 × *g* supernatant was less than 1 % of the control. With erythromycin as well as chloramphenicol the inhibition of cycloheximide-insensitive protein synthesis amounted, however, only to about 60–80 %. Both antibiotics together led to an inhibition of about 90 % of the mitochondrial protein synthesis after a preincubation time of 15 min. The effect of the two antibiotics is apparently additive. (This finding should not be interpreted as implying a certain number of sites of action for the two antibiotics. The increase in inhibition may be due to transport phenomena as well).

In order to get a maximum difference in labelling of the products of mitochondrial and cytoplasmic protein synthesizing systems, one part of the culture was thus inhibited by cycloheximide, the rest by erythromycin alone or erythromycin *plus* chloramphenicol.

The incorporation kinetics for respiring cells in the presence of cycloheximide[9] and erythromycin[12] have been demonstrated to be linear for about 30 min. From

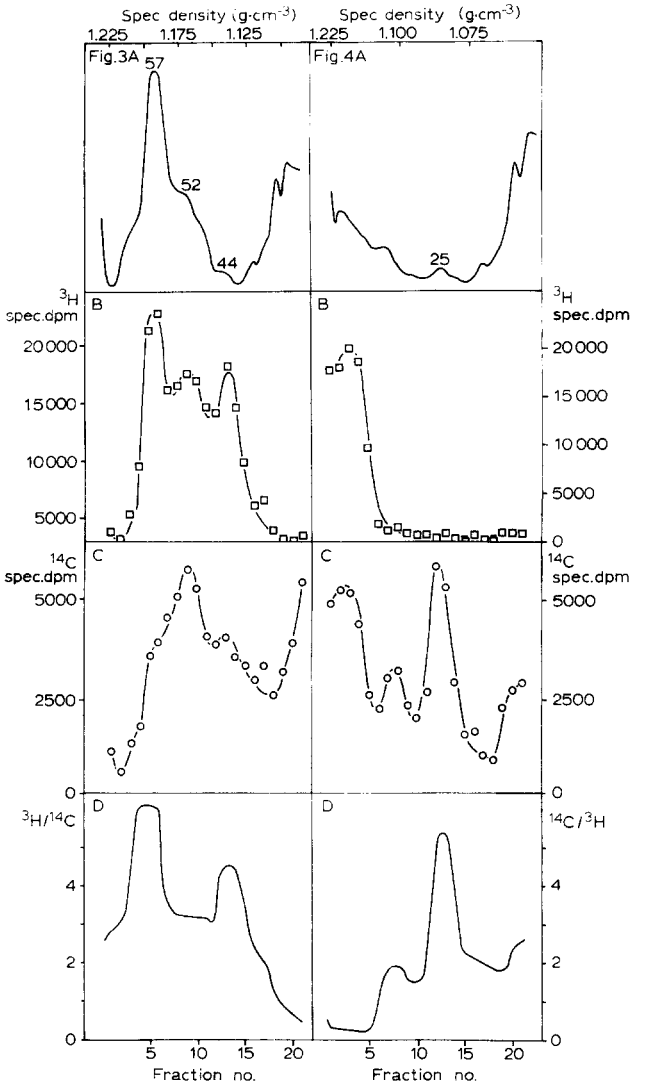
TABLE II

DISTRIBUTION OF SPECIFIC RADIOACTIVITY IN DEREPRESSED CELLS, LABELLED IN THE PRESENCE OR ABSENCE OF CYCLOHEXIMIDE

Mitochondria have been prepared by lysis of spheroplasts and purification of the crude mitochondrial fraction by sucrose gradient centrifugation. Labelling was performed as described under Materials and Methods. ^{3}H radioactivity is expressed as dpm per mg protein.

	Cycloheximide:	
	−	+
1. Cytoplasm, 155000 × *g* supernatant	28 000	240
2. Mitochondria	38 300	4380
3. Submitochondrial particles	42 200	8800
4. Soluble matrix, 155000 × *g* supernatant of 3	31 000	518

Table II it is evident that the inhibition of precursor incorporation into supernatant protein by cycloheximide amounts to about 99 %. Furthermore, it is evident that the inhibition by cycloheximide in the soluble matrix obtained from the 155000 × *g* supernatant of the submitochondrial particles following the first sonication pulses (see Materials and Methods) is of the same order of magnitude as in the cytoplasm.



Figs 3 and 4. Elution diagrams and distribution of radioactivity in the respective mitochondrial pellets (see Materials and Methods). The fraction numbers above the various peaks of the spectrophotometric traces (A) correspond to the percentage of sucrose, at which the fractions band.

Fig. 3. 15000 × *g* pellet. (A) Spectrophotometric tracing at 420 nm. (B) spec. act. of ^{3}H (dpm), labelling was in the presence of cycloheximide. (C) Spec. act. of ^{14}C (dpm), protein was labelled in the presence of erythromycin. (D) $^{3}H/^{14}C$ ratio.

Fig. 4. 155000 × *g* pellet. (A) Absorbance at 420 nm. (B) spec. act. of ^{3}H (dpm), labelling was in the presence of cycloheximide. (C) Spec. act. of ^{14}C (dpm), protein was labelled in the presence of erythromycin. (D) $^{14}C/^{3}H$ ratio.

The specific radioactivity in the mitochondrial fractions was regarded as high enough for membrane separation experiments. Labelling of the products of both cytoplasmic and mitochondrial protein synthesis and mixing of the labelled material was found to be a good guide for the identification of the various mitochondrial subfractions obtained after membrane separation and successive sucrose gradient centrifugation. Figs 3 and 4 show the elution diagrams recorded at 420 nm, the specific radioactivities, and the $^{3}H/^{14}C$ or $^{14}C/^{3}H$ ratios. The latter are used as a measure of the ratio of mitochondrial to cytoplasmic protein synthesis. According to their specific radioactivity and to the $^{3}H/^{14}C$ ratio, four main fractions may be identified from Figs 3 and 4. The densities of these fractions are exactly the same as for enzymatically determined subfractions. Fraction 25 seems to be entirely of cytoplasmic origin. This fraction has been found to be composed of outer membranes. The ratio of cytoplasmic radioactivity to succinate:cytochrome *c* reductase activity (not shown) reaches a maximum in this fraction.

The highest mitochondrial labels, that means incorporation of precursors in the presence of cycloheximide, are found in the inner membrane and in the ruptured mitochondria fractions banding with 57 % and 44 % sucrose, respectively. Intact mitochondria banding with 52 % sucrose are labelled intermediately, as can be seen from the $^{3}H/^{14}C$ ratio in Fig. 3.

Table III shows the specific radioactivity of mitochondria and mitochondrial fractions of strain M 12 labelled *in vivo* in the presence and absence of cycloheximide. The third line shows the distribution of radioactivity after sonication, centrifugation and two washings. It can be seen from the table that incorporation of radioactive isoleucine and valine into outer membranes in the presence of cycloheximide is less than 3 % of the mitochondrial label of inner membranes. Since, however, outer membranes contain about 6 % of the succinate:cytochrome *c* reductase activity of inner membranes, the residual 3 % radioactivity in the outer membranes may be accounted for by contaminating whole mitochondria or inner membranes.

From the data given in Table III it can be furthermore concluded that the turnover of the matrix is slightly lower than that of the inner membrane while that of the outer membrane is nearly two times higher.

The portion of protein provided by the mitochondrial protein synthesizing system is at least 13.5 % in intact mitochondria and nearly 40 % in pure inner membranes. These data are in fairly good agreement with those published recently (ref. 27 and R. Schweyen and F. Kaudewitz, unpublished).

Furthermore the portions of the various mitochondrial constituents can be calculated from Table III as shown in Table IV. The percentages are in good agreement with those calculated from enzyme activities:

(1) From the average enrichment of malate dehydrogenase, succinate:cytochrome *c* reductase and cytochrome *c* oxidase in the inner membrane fraction compared to intact and sonicated mitochondria (4 experiments, 9 values) and from the protein ratio of inner and outer membranes, it can be concluded that 27–30 % of the total mitochondrial protein is contributed by the inner membrane, 45–50 % by the matrix and roughly 12 % by the outer membrane.

(2) If one compares the specific activity of kynurenine hydroxylase in outer membranes to that in whole mitochondria one will find a maximum value of 8–9 % for the outer membrane protein (six preparations).

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TABLE III

SPECIFIC RADIOACTIVITY OF MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES LABELLED IN THE PRESENCE AND ABSENCE OF CYCLOHEXIMIDE WITH AND WITHOUT SUBSEQUENT MEMBRANE SEPARATION

The inhibition by cycloheximide of the synthesis of 155000 × *g* supernatant proteins has been 99.4%. Pulse labelling period was 20 min followed by a chase. ^3H radioactivity is given as dpm per mg protein.

	Intact mitochondria		*Mitochondrial fractions after membrane separation*					
			Inner membrane		*Ruptured*		*Outer membranes*	
Cycloheximide:	−	+	−	+	−	+	−	+
Untreated	10 500	1440	10 200	1900	10 800	2080	26 900	430
Cycloheximide-resistant incorporation*	13.7%		18.6%		19.2%		1.6%	
Sonicated, centrifuged and twice washed	12 800	3620	15 200	5900	12 600	3380	27 200	570
Cycloheximide-resistant incorporation*	28.3%		38.8%		26.9%		2.1%	

* Percent of the noninhibited control.

TABLE IV

PERCENTAGES OF MITOCHONDRIAL COMPARTMENTS, CALCULATED FROM TABLE III

The calculation is based on the enrichment of the specific radioactivity of the fractions. The values have been corrected for the turnovers of the various fractions.

	Matrix + intermembrane space	*Inner + outer membrane*	*Inner membrane + matrix*	*Inner membrane*	*Difference = matrix*	*Difference = outer membrane*	*Difference = intermembrane space*
Percent of whole mitochondria	60	40	76	26	52	5	17

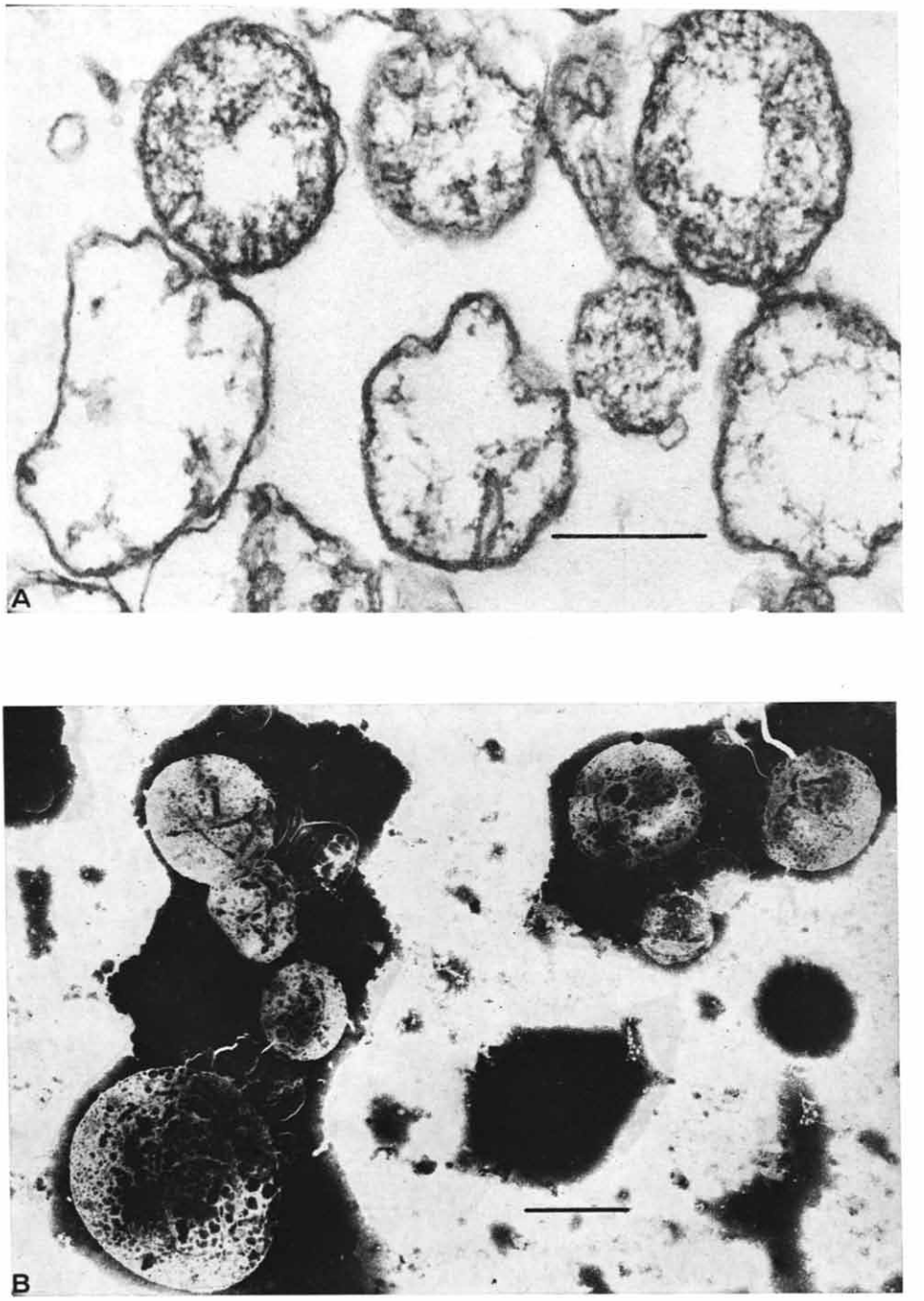

Fig. 5. Electron micrographs of intact yeast mitochondria, (A) Thin sectioned, fixed and positively stained preparation, bar 1 μm. (B) Specimen spread on a protein surface film, negatively stained, bar 1 μm.

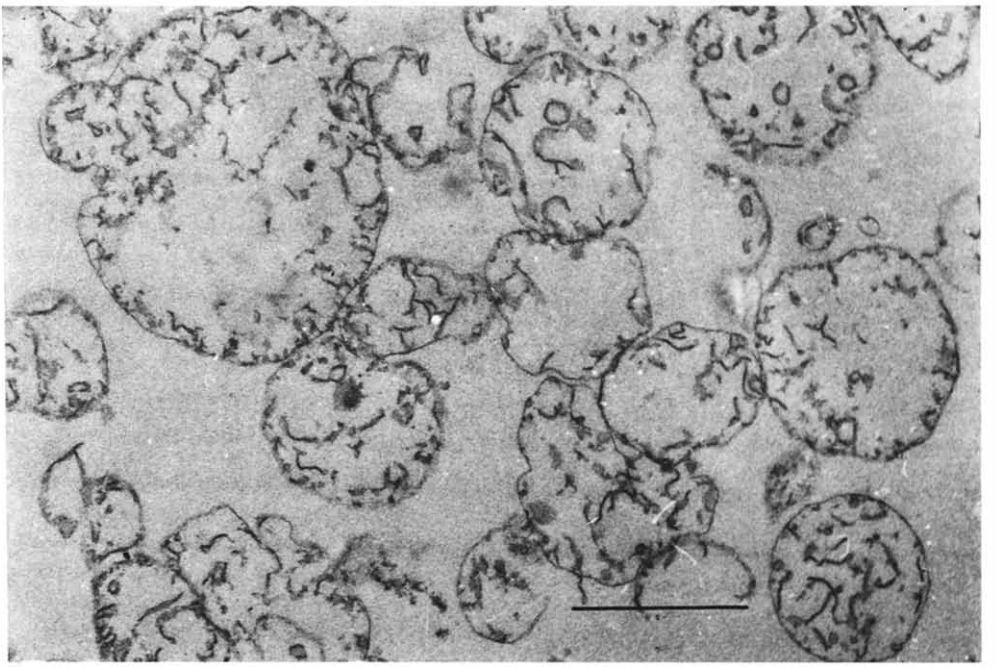

Fig. 6. Thin section of inner membranes, fixed and positively stained, bar 1 μm.

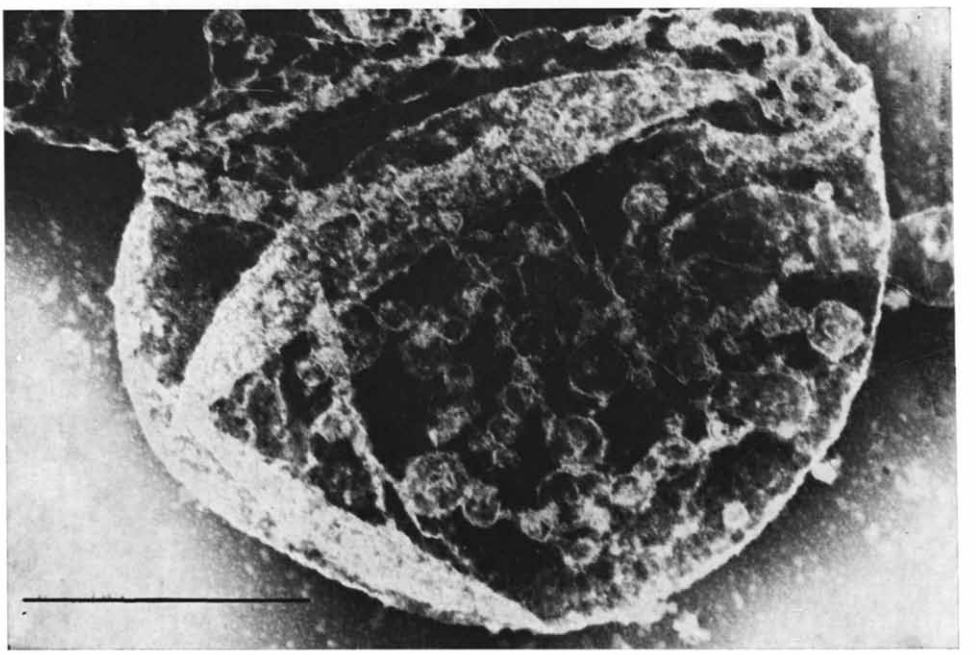

Fig. 7. Micrograph of ruptured mitochondria, negatively stained, bar 1 μm.

The discrepancy in the total of about 15 % may be due to the loss of soluble material of the intermembrane space including some lipoproteins during the membrane separation procedure.

Electron microscopy

The morphological appearance of the main fractions was checked by electron microscopy using thin sections of intact mitochondria and inner membranes. A negative staining technique was applied to both inner and outer membranes after spreading of the fraction on a protein surface film (*cf.* Materials and Methods). In intact mitochondria prepared according to both techniques a double membrane system enclosing the matrix space can be seen (see Figs 5A and 5B). Thin-sectioned inner membrane specimens show only few outer membranes attached (see Fig. 6). The inner fine structure is rather well preserved. Fractions containing ruptured mitochondria (Fraction 44) show double membrane structure and after spreading

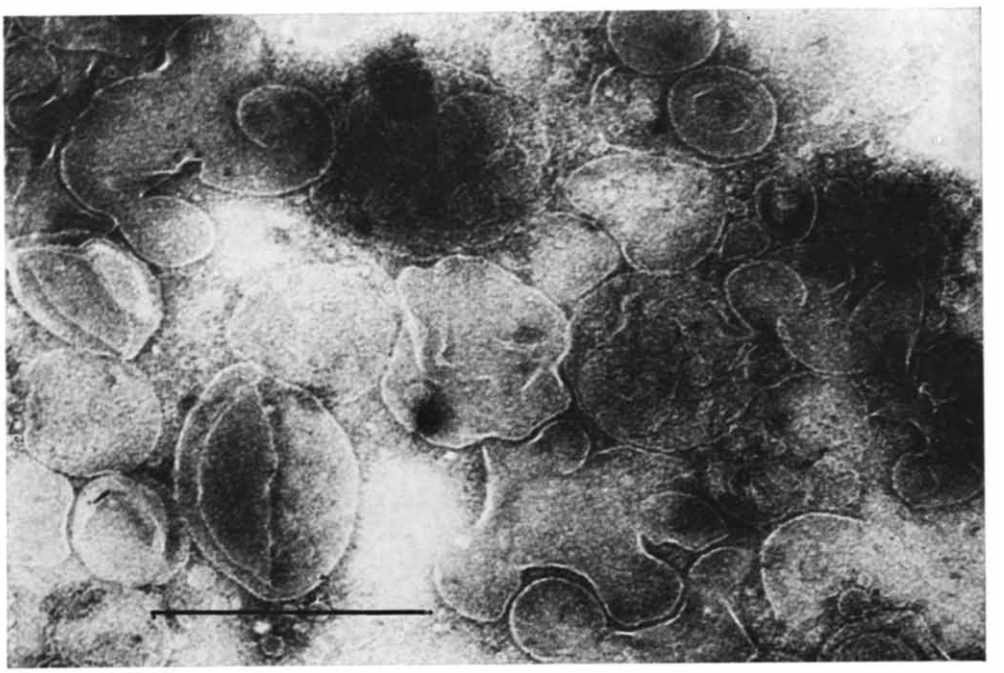

Fig. 8. Micrograph of outer membranes, prefixed and negatively stained, bar 1 μm.

permit a view into the interior cristael structure of the yeast mitochondria (Fig. 7).

Negatively stained preparations of outer membranes look like smooth empty vesicular envelopes (Fig. 8) similar to the "folded bags" obtained by Parsons *et al.*[1] from outer membranes of guinea pig liver mitochondria after prefixation with osmium tetroxide and negative staining.

DISCUSSION

Membrane separation has been achieved according to the following criteria:

(1) The inner membrane fraction contained the enzymes both of the respiratory chain and of the soluble matrix, while adenylate kinase and kynurenine hydroxylase were absent. The electron micrograph of this fraction is essentially devoid of double membrane structures.

(2) Outer membranes show kynurenine hydroxylase activity at least ten times enriched but are essentially free of adenylate kinase and of the enzymes of the inner membrane and matrix (Table I). The outer membranes are characterized by their enzymatic properties as well as by the empty envelope appearance of this fraction in prefixed negatively stained micrographs.

The experiments described in this paper were performed with highly purified mitochondria. These were prepared by lysis of spheroplasts after enzymatic digestion of the cell walls of logarithmically growing cells. This method causes the least structural damage as shown by electron micrographs prior to membrane separation. Mitochondria prepared according to this method contain all of the soluble matrix enzymes, *i.e.* malate dehydrogenase, isocitrate dehydrogenase (NADP), and glutamate dehydrogenase. The ratio of the specific activities of malate dehydrogenase to succinate:cytochrome *c* reductase in most preparations of washed intact mitochondria exceeds 100. The integrity of the mitochondria was further shown by the exhibition of a respiratory control of 1.40–1.58 and a P/O ratio of 1.89–2.04 with succinate as substrate.

The rupture caused by hypotonic swelling and shrinking[2] or by digitonin treatment[3] could be diminished by an isotonic swelling. Consequently the quantity of outer and inner membranes was enhanced mainly at the expense of ruptured mitochondria.

Kynurenine hydroxylase is bound exclusively to the outer membrane of yeast mitochondria. There is only little activity found with the inner membrane, microsomal or soluble fractions. During the separation procedure 1100 units of kynurenine hydroxylase activity were solubilized while 4600 units have been found to be bound to the outer membrane in one experiment (1 unit = formation of 1 pmole 3-hydroxykynurenine per min).

Mutual contamination of the various fractions was found to be low as judged from the specific activities of matrix and membrane-bound enzyme activities after recentrifugation and from the electron micrographs. The percentage based on protein content found for inner membrane, outer membrane and matrix *plus* intermembrane space of 27–30 %, 8 % and 62–65 %, respectively, is consistent with the results obtained from double labelling experiments and is of a similar order of magnitude as has been calculated for rat liver mitochondria[28].

From the comparison of sonicated inner membrane fractions labelled in the presence and absence of cycloheximide, it is concluded that at least one third of the protein of the inner membrane of yeast mitochondria is provided by the mitochondrial protein synthesizing system. It should be stressed, however, that these data have been found with an *in vivo* system poisoned with antibiotics. They may therefore not reliably reflect the quantitative contribution of the mitochondrial protein syn-

thesis to mitochondrial genesis in noninhibited cells. Up to now there is no definite idea about structure and function of these proteins.

Attempts to separate mitochondrial membranes of repressed yeast cells have hitherto not been successful. The experiments reported here have been performed with derepressed, respiring mitochondria. The results of the experiments strengthen the view that at least 96–97 %, but probably all of the outer membrane as well as the matrix proteins of yeast mitochondria are provided by the cytoplasmic ribosomal protein synthesizing system, as has been found earlier for mitochondria of mammalian liver[13,29] and *Neurospora crassa*[14]. It seems unlikely that the incorporation of mitochondrially synthesized precursors into the outer mitochondrial membrane should depend on the presence of a cytoplasmically synthesized integrational protein. In this case we have to accept the existence of a pool of a size greater than zero for the integrational protein. Thus in a pulse labelling experiment we might expect to find mitochondrially synthesized precursors integrated into the outer membrane in spite of the presence of cycloheximide. However, the experiments, described in this paper, will not prove the nonexistence of mitochondrially governed activator or repressor proteins which might be necessary for the regulation and correlation of the outer membrane synthesis and which might not be integrated themselves.

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REFERENCES

1 D. F. Parsons, G. R. Williams and B. Chance, *Ann. N.Y. Acad. Sci.*, 137 (1966) 643.
2 G. L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, *J. Cell Biol.*, 32 (1967) 415.
3 C. Schnaitman and J. W. Greenawalt, *J. Cell Biol.*, 38 (1968) 158.
4 J. W. Greenawalt and C. Schnaitman, *J. Cell Biol.*, 46 (1970) 173.
5 H. Okamoto, S. Yamamoto, M. Nozaki and O. Hayaishi, *Biochem. Biophys. Res. Commun.*, 26 (1967) 309.
6 M. R. Siegel and H. D. Sisler, *Biochim. Biophys. Acta*, 103 (1965) 558.
7 W. Sebald, T. Bücher, B. Olbrich and F. Kaudewitz, *FEBS Lett.*, 1 (1968) 235.
8 W. Sebald, T. Hofstötter, D. Hacker and T. Bücher, *FEBS Lett.*, 2 (1969) 177.
9 R. Schweyen and F. Kaudewitz, *Biochem. Biophys. Res. Commun.*, 38 (1970) 728.
10 G. D. Clark-Walker and A. W. Linnane, *Biochem. Biophys. Res. Commun.*, 25 (1966) 8.
11 A. W. Linnane, A. J. Lamb, C. Christodoulou and H. B. Lukins, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 1288.
12 R. Michel, R. Schweyen and F. Kaudewitz, *Mol. Gen. Genet.*, 111 (1971) 235.
13 W. Neupert, D. Brdiczka and T. Bücher, *Biochem. Biophys. Res. Commun.*, 27 (1967) 488.
14 W. Neupert and G. D. Ludwig, *Eur. J. Biochem.*, 19 (1971) 523.
15 R. Schweyen and F. Kaudewitz, *Biochem. Biophys. Res. Commun.*, 38 (1970) 728.
16 T. Ohnishi and B. Hagihara, *J. Biochem. Jap.*, 56 (1964) 484.
17 G. v. Jagow and M. Klingenberg, *Eur. J. Biochem.*, 13 (1970) 583.
18 L. Smith and H. Conrad, *Arch. Biochem. Biophys.*, 63 (1956) 403.
19 S. Ochoa, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 1, Academic Press, New York, (1955), p. 735.
20 O. Arrigoni and T. P. Singer, *Nature*, 193 (1962) 1256.
21 T. Yonetani, in R. W. Estabrook and M. E. Pullmann, *Methods in Enzymology*, Vol. 10, Academic Press, New York, (1967), p. 332.

22 B. S. S. Masters, C. H. Williams and H. Kamin, in R. W. Estabrook and M. E. Pullman, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 565.
23 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
24 L. J. Wickerham, *J. Bacteriol.*, 52 (1946) 293.
25 J. L. Simkin and T. S. Work, *Biochem. J.*, 65 (1957) 307.
26 C. W. Tabor, H. Tabor and S. M. Rosenthal, *J. Biol. Chem.*, 208 (1954) 645.
27 E. S. Hawley and J. W. Greenawalt, *J. Biol. Chem.*, 245 (1970) 3574.
28 G. Brunner and T. Bücher, *FEBS Lett.*, 6 (1970) 105.
29 D. S. Beattie, R. E. Basford and S. B. Koritz, *Biochemistry*, 6 (1967) 3099.