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Mitochondrial Translation Products before and after Integration into the Mitochondrial Membrane in *Neurospora crassa*

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1. Nascent translation products on mitochondrial ribosomes were selectively labeled *in vivo* in the presence of cycloheximide with radioactive leucine. They were isolated together with the ribosomes.

2. The labeled polypeptides show a high tendency to aggregate and can only be kept in solution in the presence of detergents such as dodecylsulfate. Also, mitochondrial ribosomes carrying nascent peptide chains easily form aggregates.

3. The polypeptides adhering to mitochondrial monomeric ribosomes differ from those adhering to polymeric ribosomes. Gel electrophoresis in the presence if dodecylsulfate shows for the peptidyl transfer RNA products at the monomer, an apparent molecular weight of 27000. After removing the transfer RNA, an apparent molecular weight of less than 10000 is registered. The peptides adhering to mitochondrial polymeric ribosomes display a broad range of apparent molecular weights. In contrast, translation products associated with cytoplasmic monomeric and polymeric ribosomes all show quite disperse molecular weights.

4. Using gel-chromatographic analysis no difference in the elution characteristics between translation products associated with mitochondrial monomeric and polymeric ribosomes was found. In both cases apparent molecular weights of about 11000 were obtained.

5. A kinetic study of the appearance of mitochondrial translation products in the mitochondrial membrane was carried out. A conversion process of products with lower apparent molecular weights to those with higher apparent molecular weights is observed. This suggests that mitochondrial ribosomes form polypeptides which are modified during or after integration into the membrane.

6. The hypothesis is discussed that mitochondria possess their own system of transcription and translation, because the hydrophobic nature of the translation products makes it necessary that they are formed inside the inner mitochondrial membrane, into which they are integrated.

It is not yet understood, why the mitochondrion possesses a specific apparatus of transcription and translation (for reviews, see [1–5]). As a matter of fact, some 100 different proteins are formed at the cytoplasmic ribosomes for the intramitochondrial transcription and translation machinery [6–12]. This machinery produces a few polypeptides which are integrated into the inner mitochondrial membrane [1–5].

An explanation for this astonishing situation may residue in the nature and properties of the polypeptides formed within the mitochondrion.

In the following we report on the isolation of mitochondrial ribosomes and the characterisation of the adhering peptide chains in comparison to cytoplasmic ribosomes. Furthermore, pulse-chase experiments were carried out to follow the appearance of mitochondrial translation products in the mitochondrial membrane.

The hypothesis is brought up that the mitochondrial translation products, due to their hydrophobic character, must be transported to the inner membrane from the matrix side. This is thought to be the reason why the eucaryotic cell has a second, the intramitochondrial protein synthetic machinery.

Abbreviation. Butyl-PBD, 2-(4-*t*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole.

MATERIALS AND METHODS

Preparation of Mitochondria and Ribosomes

Mitochondria were isolated from *Neurospora* cells (wild-type 74A) grown for 18 h as described by Weiss *et al.* [13]. Mitochondrial ribosomes were prepared after lysis of mitochondria in a buffer A con-

taining 0.1 M NH_4Cl, 0.01 M $MgCl_2$ and 0.01 M Tris-HCl pH 7.5 and 1% Triton X-100 as described [14], with the modification that the lysate was placed on a layer of 1.5 ml 1.4 M sucrose in buffer A and centrifuged for 2 h at 144000 × *g*.

For preparation of cytoplasmic ribosomes, the cells were disrupted in a grinding mill [13] with buffer A containing 0.44 M sucrose. The homogenate was centrifuged for 20 min at 17000 × *g*. To the resulting supernatant, Triton X-100 was added (final concn. 1%). After a clarifying spin (15 min, 27000 × *g*) the lysate was centrifuged on a layer of 1.5 ml 1.4 M sucrose in buffer A for 2 h at 144000 × *g*.

Sucrose-density-gradient centrifugation of mitochondrial and cytoplasmic ribosomes was carried out as described previously [14]. Gradients were monitored with a Zeiss PMQ II photometer, using a flow cell (50-µl volume, 1-cm path-length). Radioactivity was measured in fractions automatically collected with a LKB Ultrorac.

Mitochondrial membranes were prepared after extraction of soluble proteins from mitochondria by sonication as described by Sebald *et al.* [15].

Preparation of Crude Mitochondrial and Cytoplasmic G-Factors

To prepare crude mitochondrial G-factor a mitochondrial suspension in buffer A (3 mg protein/ml) was sonicated for 200 s (Branson, sonifier model S75, 6 amps output). The suspension was then centrifuged for 2 h at 144000 × *g*. From the supernatant a crude preparation of G-factor was obtained with the method of Grandi and Küntzel [16].

For the preparation of crude cytoplasmic G-factor, *Neurospora* cells were disrupted in buffer A containing 0.44 M sucrose. After sedimentation of cell walls and of mitochondria, the supernatant was centrifuged for 3 h at 144000 × *g*. Further treatment of the supernatant was the same as in the method used for the mitochondrial G-factor.

Labeling Procedures

In order to selectively label mitochondrial translation products *in vivo*, *Neurospora* cells were first incubated for 2.5 min with cycloheximide (0.1 mg/ml) (C. Roth OHG, Karlsruhe, Germany). Then 1 µCi/ml L-[^{3}H]leucine (specific activity 52 Ci/mmol; Radiochemical Centre, Amersham, England) was added. For the labeling of nascent peptide chains on mitochondrial ribosomes, the cells were rapidly cooled to 0 °C after 1.5 min. For the labeling of mitochondrial translation products in the mitochondrial membrane, a chase of unlabeled L-leucine (final concn 3 mM) was applied after 1.5 min, which lasted for 0, 15, 30 and 60 min. After the chase periods the cells were cooled to 0 °C.

Incubation of isolated mitochondria with L-[^{3}H] leucine (5 µCi/ml) was carried out in a medium described by Sebald *et al.* [15].

Labeling *in vivo* of nascent peptides on cytoplasmic ribosomes was achieved by adding L-[^{3}H] leucine (2 µCi/ml) to a *Neurospora* culture. After 35 s, the cells were rapidly cooled to 0 °C.

Polyacrylamide-Gel Electrophoresis

After dilution with buffer A, gradient fractions were centrifuged for 2 h at 144000 × *g* to sediment the ribosomal particles. The resulting pellets were resuspended in 0.1 M Tris-HCl pH 8.0, and sodium dodecylsulfate was added (final concn 1%). The samples were incubated at 37 °C for 2 h with and without 50 µg/ml pancreatic ribonuclease (Boehringer Mannheim GmbH, Mannheim, Germany). Aliquots of 20 µl were subjected to electrophoresis on 15% polyacrylamide gels containing 0.5% sodium dodecylsulfate as described by Weiss *et al.* [17].

The pellets of mitochondrial membranes were dissolved in 0.1 M Tris-HCl pH 8.0, 1% sodium dodecylsulfate and kept at 37 °C for 2 h. Samples of 10–20 µl (50–100 µg protein) were subjected to the same electrophoretic procedure.

Parallel to the samples, a mixture of the following marker proteins was run on the same gel: bovine serum albumin, mol. wt, 64000; enolase, mol. wt, 43000; triosephasphate isomerase, mol. wt, 27000; lactoglobulin A, mol. wt, 17500; cytochrome *c*, mol. wt, 11600; and glucagon, mol. wt, 3600. The gels were stained with 0.25% coomassie brilliant blue R-250 (Serva Entwicklungslabor, Heidelberg, Germany) in methanol–water–acetic acid (5:5:1, v/v/v). Destaining of the gels was performed in the same solvent.

Gel-Filtration on Sephadex G-200

Sediments of monomeric and polymeric ribosomes (see previous section) were subjected to two different treatments. One portion was incubated with pancreatic ribonuclease in the presence of dodecylsulfate as described above. To a second portion, sodium dodecylsulfate (final concn 1%) was added and then trypsin (10 mg/ml). Incubation at 32 °C lasted 6 h. Samples from both procedures (0.1 ml) were applied to columns (0.4 × 100 cm) filled with Sephadex G-200 (Pharmacia, Uppsala, Sweden), equilibrated with 0.1 M Tris-HCl pH 8.0, 0.5% sodium dodecylsulfate. Elution was carried out with equilibration buffer at a rate of 0.25 ml/h. The columns were calibrated by cochromatography of dextran blue, cytochrome *c* and ^{14}C-labeled leucine.

Determination of Radioactivity

Radioactivity was measured in a Packard liquid scintillation counter using butyl-PBD scintillator

(6 g/l in toluene—2-methoxyethanol, 3:2, v/v). For determining radioactivity in gels, these were cut into slices of 1 mm. The slices were shaken with 0.5 m 0.5% sodium dodecylsulfate, 0.1 M Tris-HCl pH 8.0, in counting vials at 60 °C for 12 h before adding scintillation liquid.

RESULTS

Labeling of Nascent Polypeptide Chains

Specific labeling *in vivo* of nascent polypeptide chains on mitochondrial ribosomes was achieved by incubating *Neurospora* cells for 2.5 min with cycloheximide and then for 1.5 min with radioactive leucine. Cycloheximide has been shown to inhibit cytoplasmic translation selectively [18—20]. The synthesis of mitochondrial ribosomal proteins is abolished under the action of this antibiotic [7—9].

Fig. 1 A presents a sucrose-density-gradient profile of mitochondrial ribosomes from such cells. In the pattern of absorbance at 260 nm a peak corresponding to the monomeric ribosome is prominent. In the lower part of the tube, peaks corresponding to di-, tri- and tetameric ribosomes can be distinguished. Free subunits are present. Fig. 1 A also shows the distribution of radioactivity in the gradient. A distinct peak corresponds to the monomeric ribosomes, but most of the radioactivity is however found associated with polymeric ribosomes. In different experiments, between 10 and 50% of the total radioactivity in the gradient is found associated with the monomeric ribosomes. Clearly, the ratio of radioactivity to absorbance at 260 nm in the dimer and trimer exceeds by far that in the monomer.

A second portion of mitochondria, equivalent to that from which these ribosomes were isolated, was incubated with puromycin, in a medium optimal for amino acid incorporation [15], prior to the isolation of ribosomes. When ribosomes from these mitochondria were subjected to analysis by density-gradient centrifugation, virtually no radioactivity was detected in the gradient (not shown here). This demonstrates that all radioactivity, present at the mitochondrial ribosomes, represents nascent translation products.

Cytoplasmic ribosomes were isolated from cells after pulse labeling for 45 s with radioactive leucine. Density-gradient analysis of these ribosomes is shown in Fig. 1 E. The absorbance at 260 nm profile shows monomeric ribosomes and an appreciable amount of polymeric ribosomes. The ratio of radioactivity to absorbance at 260 nm is constant in monomeric and polymeric ribosomes. Addition of puromycin to the cells after the 45-s radioactivity pulse results in the removal of more than 80% of the radioactivity associated with the ribosomes. Evidently, the radioactive pulse essentially produces labeling of the nascent translation products.

Mitochondrial and cytoplasmic ribosomes display a marked difference in their tendency to aggregate. After sedimentation from cell homogenates, cytoplasmic ribosomes can easily be resuspended. The formation of heavy aggregates is negligible, as judged from measurements of radioactivity, representing nascent peptide chains and of absorbance at 260 nm, after resuspension. Upon gradient centrifugation, cytoplasmic ribosomes are completely recovered in the gradient. No pellet at the bottom of the centrifuge tube is observed.

In contrast, after sedimentation from lysates of mitochondria, mitochondrial ribosomes can never be completely resuspended. It is difficult to measure the loss of radioactivity in this step, since some membraneous material is always present in preparations of crude mitochondrial ribosomes. On the basis of a rough calculation, about 20% of the ribosomes are lost. These form highly aggregated particles which cannot be resuspended. About 30—40% of the total radioactivity, sedimented from mitochondrial lysates is found associated with this fraction. Furthermore, up to 20% of the absorbance at 260 nm of the material applied to the density gradient, and up to 40% of the radioactivity are found at the bottom of the centrifuge tube after centrifugation. 20% of the radioactivity, applied to the gradient, are attached to the sides of the centrifuge tube.

When nascent peptide chains are removed from the mitochondrial ribosomes by incubating isolated mitochondria with puromycin, the aggregation of these ribosomes is greatly reduced. The amount of highly aggregated ribosomes in a suspension of crude ribosomes is decreased to 30% compared to the control. After gradient centrifugation the amount of ribosomes in the pellet is reduced to 10% of that in the control. Furthermore, no more radioactivity can be detected at the bottom of the centrifuge tube.

These observations demonstrate the strong tendency of mitochondrial ribosomes to aggregate, especially when they carry nascent peptide chains.

Properties of Nascent Peptide Chains on Cytoplasmic and Mitochondrial Ribosomes

In Fig. 1 B and F gradient profiles of mitochondrial and cytoplasmic ribosomes, which were kept at 32 °C for 10 min, are shown. With cytoplasmic ribosomes, a partial conversion of polymeric into monomeric forms is observed. This probably is caused by an endogenous ribonuclease. A quite different result is obtained with mitochondrial ribosomes. In this case, a large part of the polymers also disappears, as demonstrated by the profile of absorbance at 260 nm. The monomer peak, however, is not increased. The radioactivity in the polymer

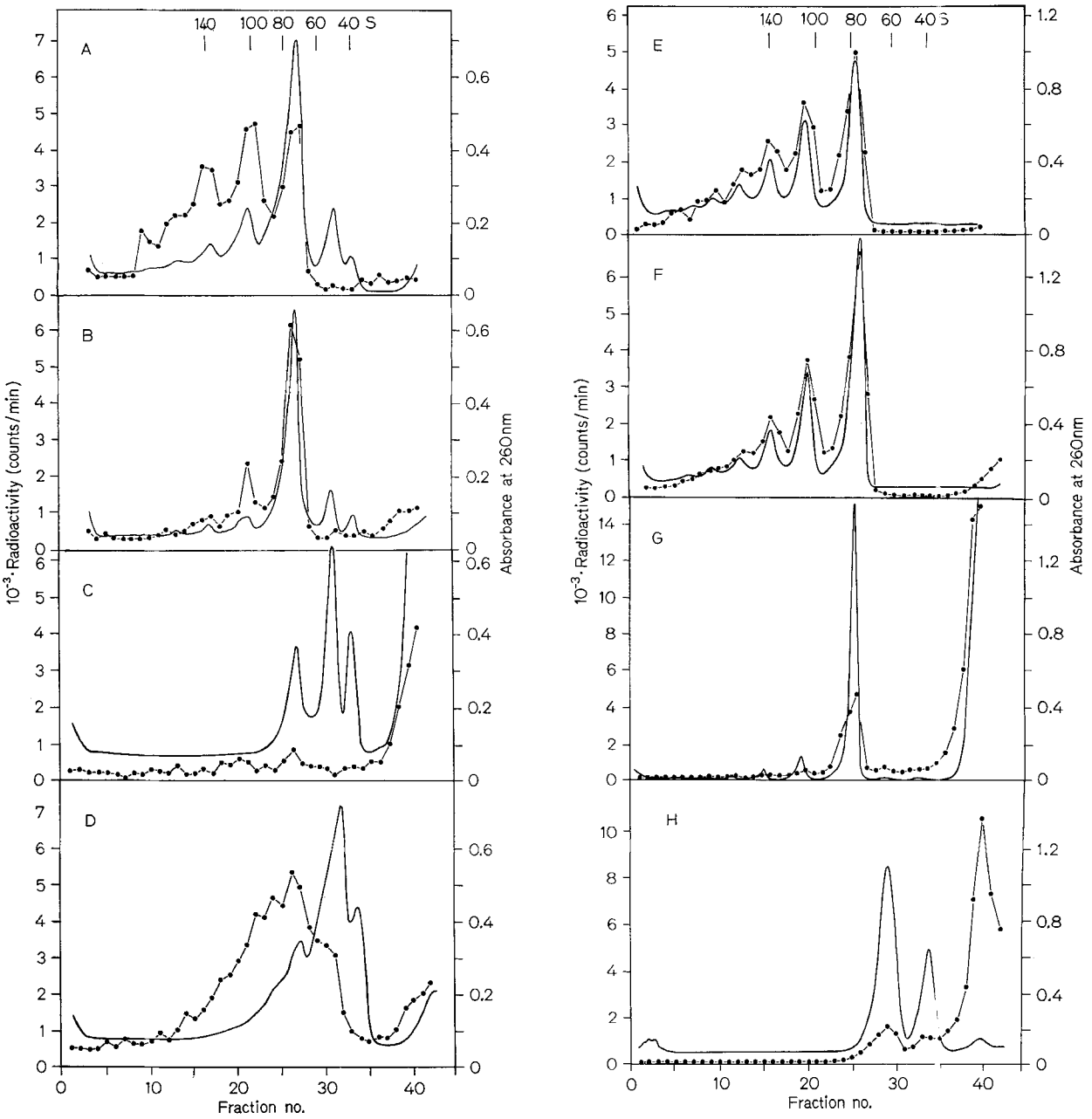


Fig. 1. *Density-gradient centrifugation of mitochondrial and cytoplasmic ribosomes.* Mitochondrial ribosomes were isolated from *Neurospora* cells labeled for 1.5 min in the presence of cycloheximide with [^{3}H]leucine. Cytoplasmic ribosomes were isolated from cells labeled for 45 s with [^{3}H]leucine. (●) Radioactivity; (——) absorbance at 260 nm. (A—D) Mitochondrial ribosomes; (E—H) cytoplasmic ribosomes; (A, E) controls; (B, F) ribosomes were kept at 32 °C for 10 min in buffer A; (C, G) ribosomes were treated in buffer A with 0.5 mM puromycin, 0.5 mM GTP, crude mitochondrial or cytoplasmic G-factor (20 μg/ml) for 10 min at 32 °C and (D, H) gradient contained a layer of 1.5 ml 7% sucrose in 0.1 mM EDTA, 0.01 M Tris-HCl pH 7.5, on top

region is greatly decreased, but in the monomer region it remains constant. The part disappearing in the polymer region is found at the bottom of the centrifuge tube.

The radioactivity associated with mitochondrial polymeric and monomeric ribosomes was followed during a 40-min incubation period at 37 °C (Fig. 2). The radioactivity at the monomer remains rather

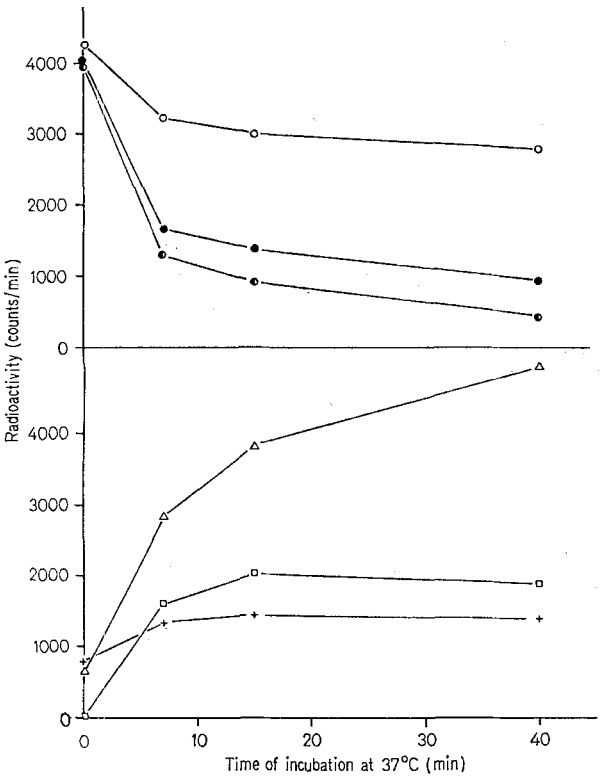


Fig. 2. *Effect of incubation of mitochondrial ribosomes in buffer A at 37 °C on the distribution of radioactivity in the sucrose-density gradient.* Mitochondrial ribosomes, labeled *in vivo* in the presence of cycloheximide with [^{3}H]leucine, were kept at 37 °C in buffer A for the time periods indicated in the figure. They were then subjected to gradient centrifugation. (○) Monomeric ribosomes; (◐) dimeric ribosomes; (●) trimeric and higher polymeric ribosomes; (△) gradient pellet; (□) wall of centrifuge tube [radioactivity in the untreated sample (8250 counts/min) was subtracted from all values] and (+) top of gradient

constant, after a slight initial decrease; in the dimer and higher polymer region, it is strongly diminished. It appears mainly in the pellet of the gradient, a smaller part becoming attached to the wall of the centrifuge tube.

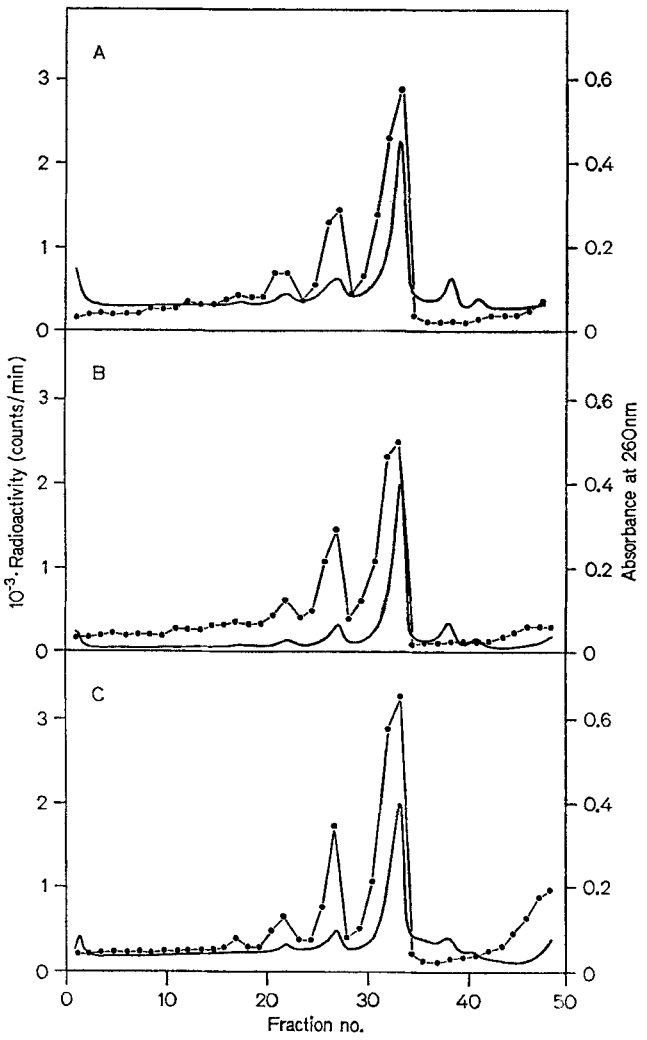
Cytoplasmic ribosomes of the same batch subjected to density-gradient centrifugation in Fig. 1 E, were incubated with puromycin in the presence of GTP and crude cytoplasmic G-factor. About 80% of the radioactivity is released from the ribosomes. Applying density-gradient centrifugation, the released radioactivity appears in the top layer of the gradient (Fig. 1 G). This again demonstrates that the radioactivity, associated with these ribosomes, largely represents nascent peptide chains. Furthermore, it shows that the puromycin peptides generated in this reaction are water-soluble components. Both conclusions are supported by the following observation: removal of magnesium ions from the ribosomes by centrifuging them through a layer of sucrose containing 0.1 mM EDTA on the top of the gradient, accomplishes a complete dissociation into subunits. This is accompanied by the release of about 80% of the radioactivity from the ribosomes. Some 20% remain with the emerging subunits (Fig. 1 H). The radioactivity liberated from the ribosomes moves into the 4-S position. This indicates peptidyl transfer RNA and shows that these components are water soluble.

The same experiments were carried out with mitochondrial ribosomes. Similar to cytoplasmic ribosomes, treatment with puromycin, GTP and crude mitochondrial G-factor removes all radioactivity from the ribosomes. However, after centrifugation only a small part of the puromycin peptides appears at the top of the gradient (Fig. 1 C). The rest is found at the bottom and at the sides of the centrifuge tube. Upon centrifugation through an EDTA-containing layer, the bulk of the mitochondrial ribosomes appear as subunits. Polymeric ribosomes are destroyed. Some of the ribosomal material still appears in the monomer region. This part, however, could represent aggregates of subunits rather than intact monomers. The absorbance profile displays several shoulders in the region of 70 to 90 S. Radioactivity cannot be traced in the 4-S region. Some is found with the large subunit. Mainly it is in the region between 70 and 100 S and cannot be associated to definite ribosomal structures.

Effect of Ribonuclease on Cytoplasmic and Mitochondrial Ribosomes

When cytoplasmic ribosomes are incubated with pancreatic ribonuclease at 0 °C for 60 min, a conversion of polymers to monomers can be observed in the absorbance profile as well as in the radioactivity profile (Fig. 3 D–F). Concentrations of ribonuclease as low as 0.1 μg/ml are sufficient to cause a notable destruction of the higher polymeric forms. After incubation at concentrations of 20 μg/ml, virtually no more polymeric forms are found in the gradient.

Mitochondrial polymeric ribosomes appear to be completely resistant at such concentrations of ribonuclease (Fig. 3 A–C). Neither the absorbance profile nor the radioactivity profile is changed. Only free mitochondrial subunits are destroyed, using the higher concentration of ribonuclease. Treatment of mitochondrial ribosomes with T_1 ribonuclease has the same result. A similar observation on the resistance against ribonuclease with mitochondrial ribosomes from HeLa cells has been made by Ojala and Attardi [21].



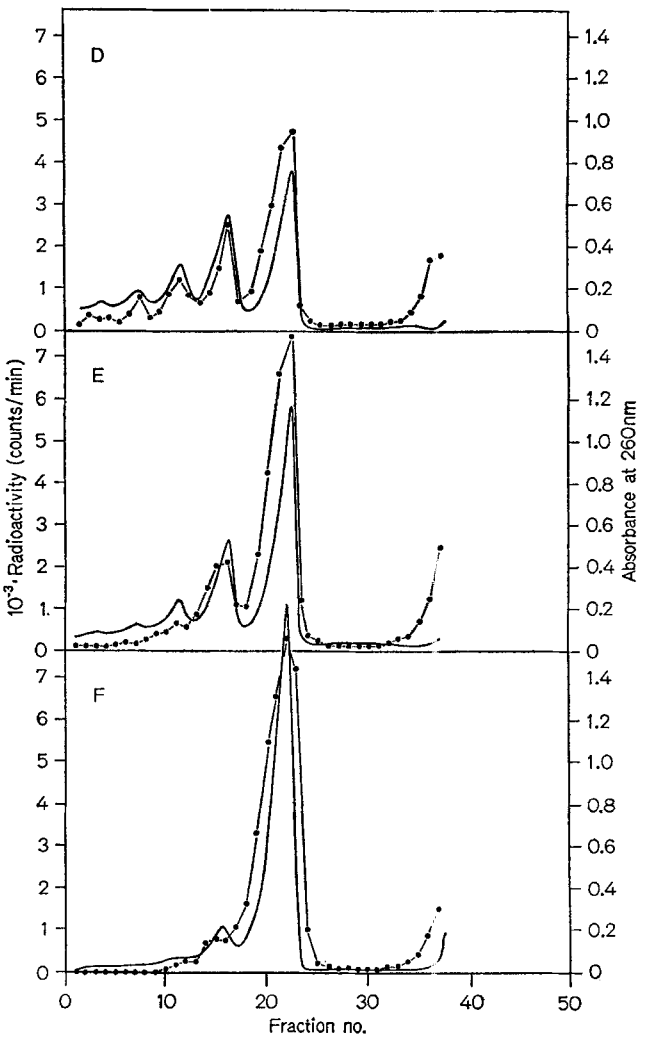


Fig. 3. *Treatment of mitochondrial and cytoplasmic ribosomes with ribonuclease.* Ribosomal suspensions in buffer A were kept at 0 °C for 60 min with and without pancreatic ribonuclease, and then subjected to gradient centrifugation. (●) Radioactivity; (——) absorbance at 260 nm. (A—C) Mitochondrial ribosomes; (D—F) cytoplasmic ribosomes; (A, D) control (without ribonuclease); (B, E) 0.5 μg/ml ribonuclease and (C, F) 20 μg/ml ribonuclease

Localisation of Mitochondrial Ribosomes Carrying Nascent Peptide Chains

The usual procedures to isolate mitochondrial ribosomes involve the application of detergents to dissolve the mitochondrial membranes, thus setting the mitochondrial ribosomes free. These methods do not allow an opinion on whether the ribosomes are bound to the inner membrane or whether they are freely distributed in the matrix. As an approach to this problem, it was investigated whether mechanical disruption of mitochondria could free the mitochondrial ribosomes.

In a first experiment mitochondria, after pulse labeling *in vitro* with radioactive leucine, were subjected to mild sonication. The sonicate was centrifuged to sediment large vesicles. This was followed by a high-speed centrifugation to sediment small vesicles and free ribosomes. Both pellets were divided into two equal portions, and one of each portion was lysed by the addition of Triton X-100. All portions were then subjected to density-gradient centrifugation. In the gradient fractions the absorbance at 260 and 280 nm, and radioactivity were measured.

The result is presented in Fig. 4. The low-speed pellet before lysis, displays a broad distribution of absorbance at 260 and 280 nm. The bulk of the radioactivity is found in the lower third of the tube. This indicates rapidly sedimenting vesicles. The radioactivity profile shows a distinct peak at

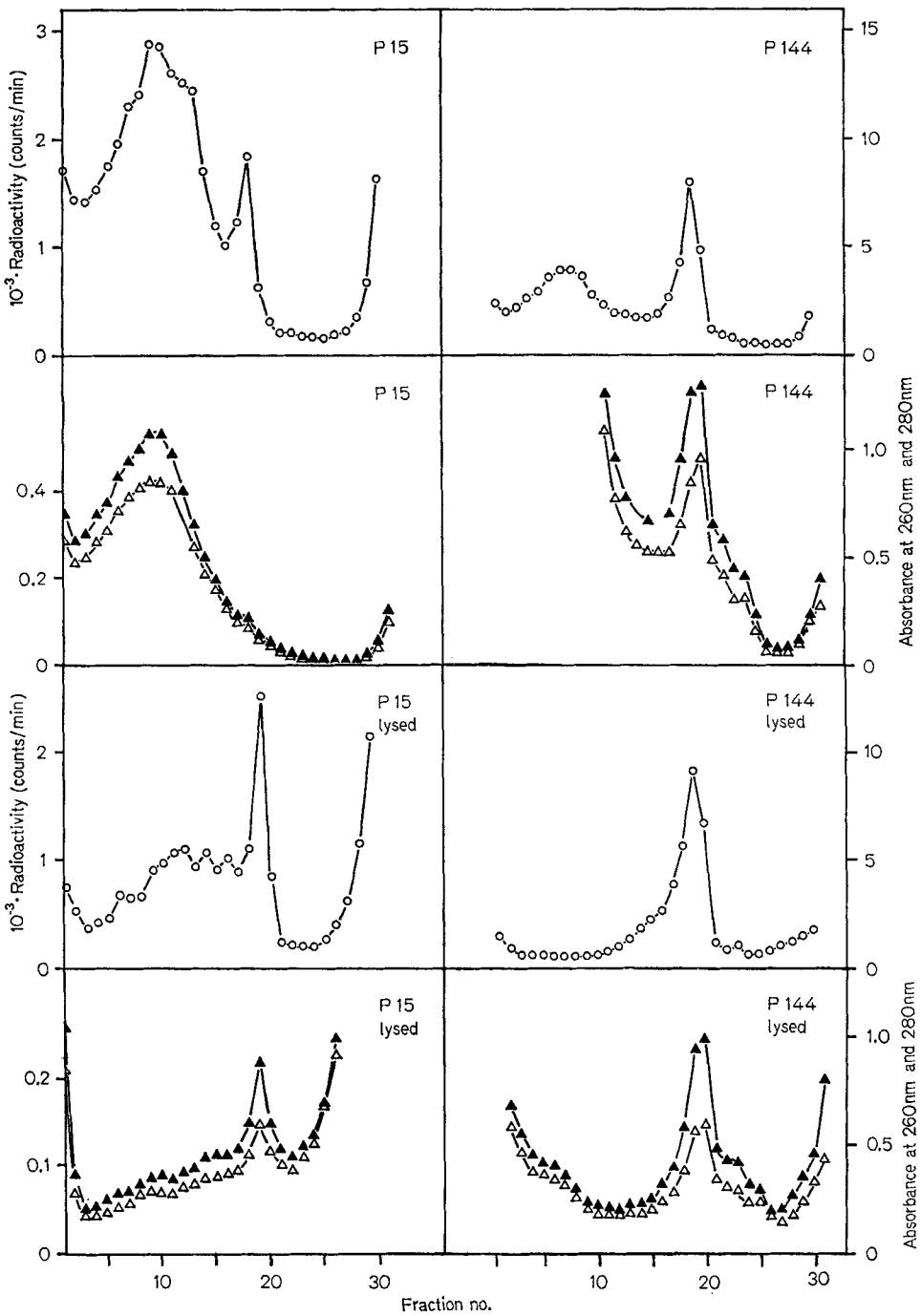


Fig. 4. *Extraction of ribosomes from mitochondria by sonication.* [^{3}H]Leucine was incorporated into isolated mitochondria. After washing, the mitochondria were exposed to sonic treatment (50 s, Branson sonifier, 3 amps output). The suspension was centrifuged for 20 min at 15000 × *g* to give the low-speed pellet (P15); the resulting supernatant was centrifuged for 1 h at 144000 × *g* to give the high-speed pellet (P144). Both sediments were divided into two equal portions. One of each was lysed by adding Triton X-100 (final concn 2.5%). All portions were then subjected to sucrose-density-gradient centrifugation. In the gradient fractions the absorbance at 260 and 280 nm and the radioactivity were measured. (○) Radioactivity; (▲) absorbance at 260 nm and (△) absorbance at 280 nm

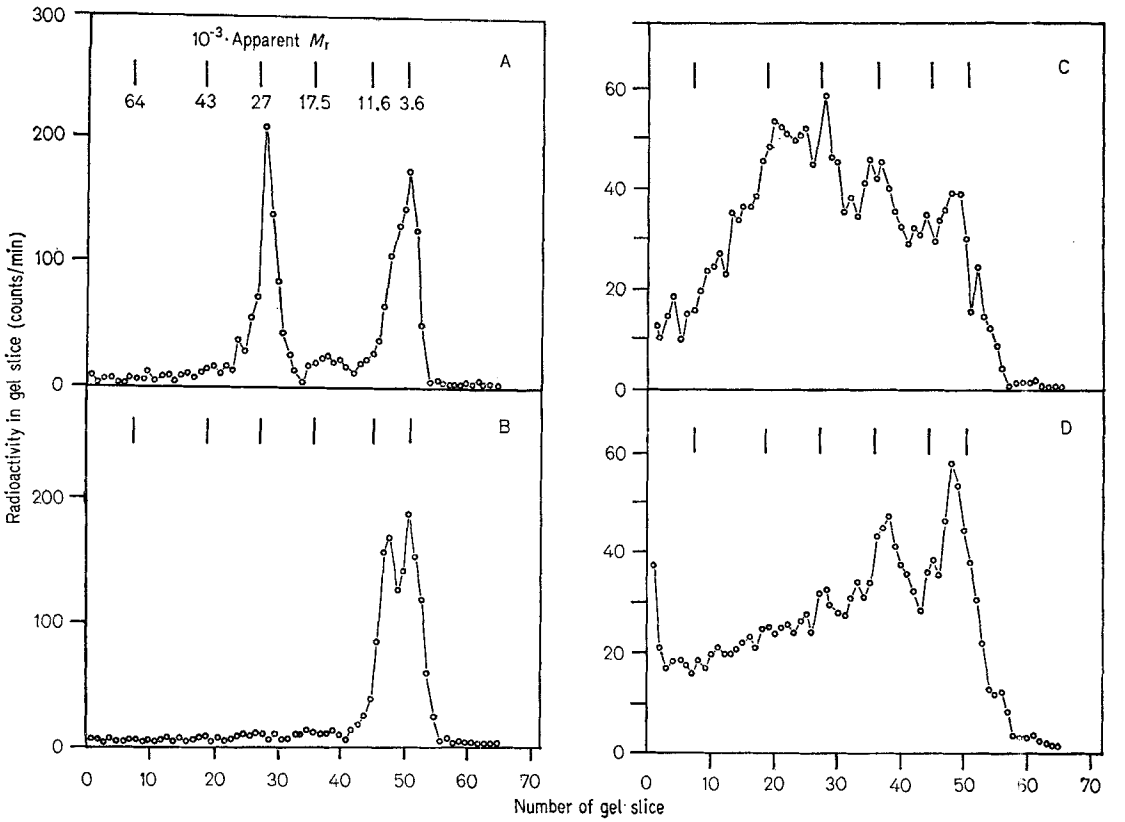


Fig. 5. *Gel-electrophoretic analysis of radioactively labeled nascent peptide chains associated with mitochondrial monomeric and polymeric ribosomes.* (A) Monomeric ribosomes; (B) monomeric ribosomes, treated with ribonuclease; (C) polymeric ribosomes and (D) polymeric ribosomes, treated with ribonuclease

the position expected for the mitochondrial monomeric ribosome. Obviously, this preparation contains free ribosomes.

After lysis of the low-speed-pellet fraction, the vesicles in the gradient almost completely disappear. A small peak at the monomer position becomes apparent in the profile of absorbance at 260 and 280 nm. In the radioactivity profile this peak also is present. It has about the same height as in the unlysed sample. This indicates that the ribosomes found in the unlysed low-speed-pellet fraction were sedimented together with but not bound to or enclosed by the vesicles.

The high-speed pellet of the sonicated mitochondrial suspension contains the bulk (more than 90%) of these mitochondrial ribosomes. This is revealed by the radioactivity profile and the profile of absorbance at 260 and 280 nm of the unlysed preparation. Again, lysis with Triton X-100 increases only slightly the amount of free ribosomes.

In a second experiment, a mitochondrial suspension was subjected to repeated freezing and thawing (15 times). About 30% of the mitocondrial ribosomes were set free. This may be considered as a good yield, since it can be expected that relatively large particles such as ribosomes, do not easily leave the mitochondrial matrix space during the short openings of the membrane.

It may be mentioned that by sonication of preparations of rat-liver endoplasmic reticulum under the same conditions, less than 10% of the membrane-bound ribosomes were set free.

The data presented here suggest that the bulk of the mitochondrial ribosomes can be set free by mechanical disruption of mitochondria. A similar observation with yeast mitochondria was reported by Green and Watson [22].

Analysis of Nascent Peptide Chains Associated with Ribosomes by Gel Electrophoresis in the Presence of Dodecylsulfate

The fractions of density gradients containing monomeric and polymeric ribosomes, labeled *in vivo* as outlined above, were separately centrifuged to sediment the particles. The resulting pellets were resuspended in Tris buffer and dodecylsulfate was added. The samples were incubated for 2 h in the presence and absence of ribonuclease. They were then subjected to electrophoresis on polyacrylamide gels with dodecylsulfate.

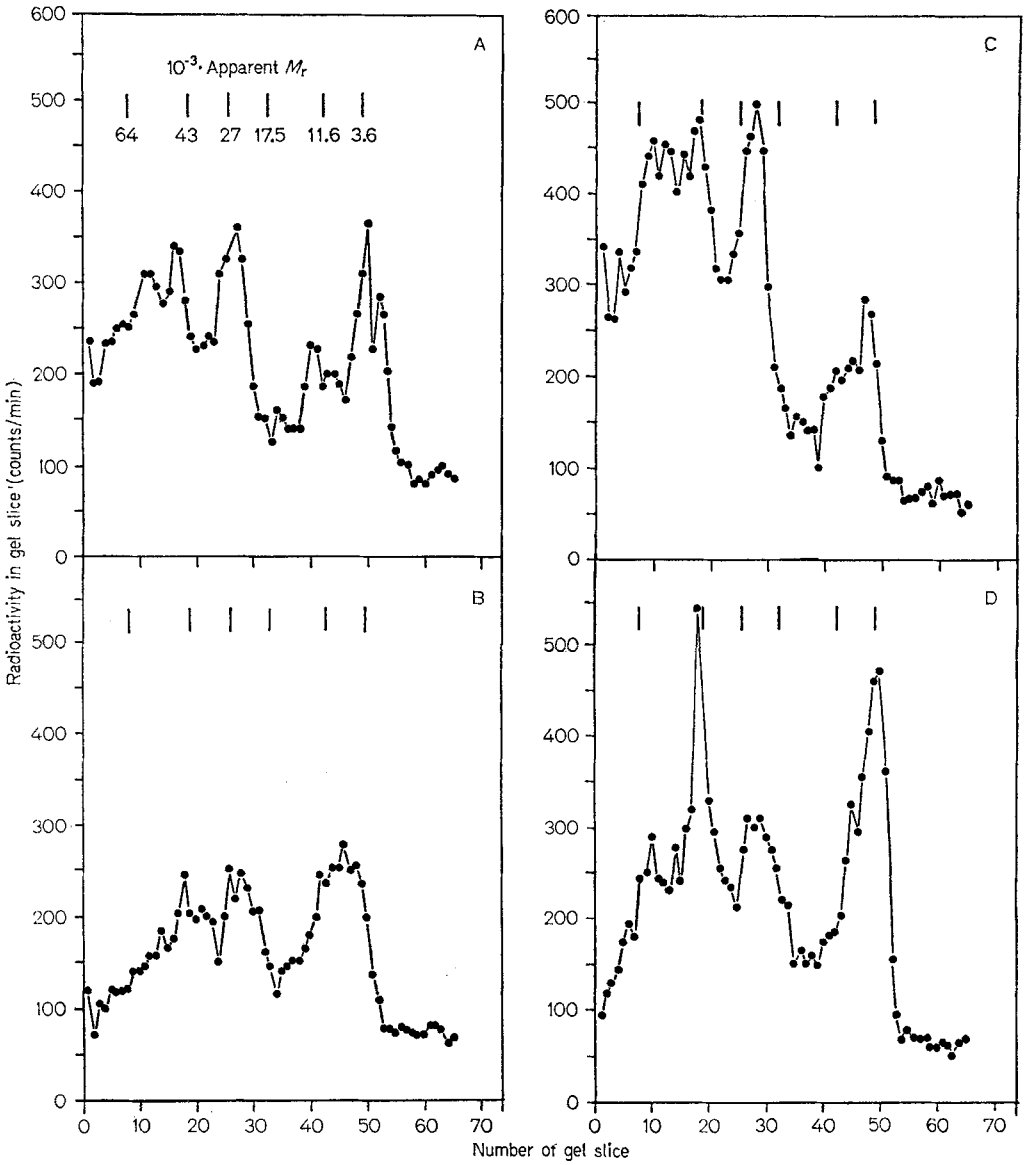


Fig. 6. *Gel-electrophoretic analysis of radioactively labeled nascent peptide chains associated with cytoplasmic monomeric and polymeric ribosomes.* (A) Monomeric ribosomes; (B) monomeric ribosomes, treated with ribonuclease; (C) polymeric ribosomes and (D) polymeric ribosomes, treated with ribonuclease

Fig. 5 demonstrates that the distribution of radioactivity on the gels of mitochondrial polymeric and monomeric ribosomes differs greatly. In the samples not treated with ribonuclease, the polymers show a broad distribution without distinct peaks (Fig. 5C). The same holds for that part of the mitochondrial ribosomes which forms a pellet in the sucrose-density gradient.

With monomeric ribosomes, essentially two sharp peaks are observed: one at a position corresponding to an apparent molecular weight of 27000, and a second one with an apparent molecular weight of 5000—10000, with a shoulder towards higher apparent molecular weights (Fig. 5A). During prolonged incubation (up to 6 h) in dodecylsulfate-containing buffer (0.1 M Tris-HCl pH 8.0) the first peak disappears. The corresponding radioactivity is found with the second peak, where a double peak is now seen. A rapid conversion of the first peak to the second one is observed, when ribosomes are dissolved in a buffer with a pH of 11. Immediate application of the monomeric ribosomes to gel electrophoresis, after dissolving them in cold buffer, mainly produces the first peak.

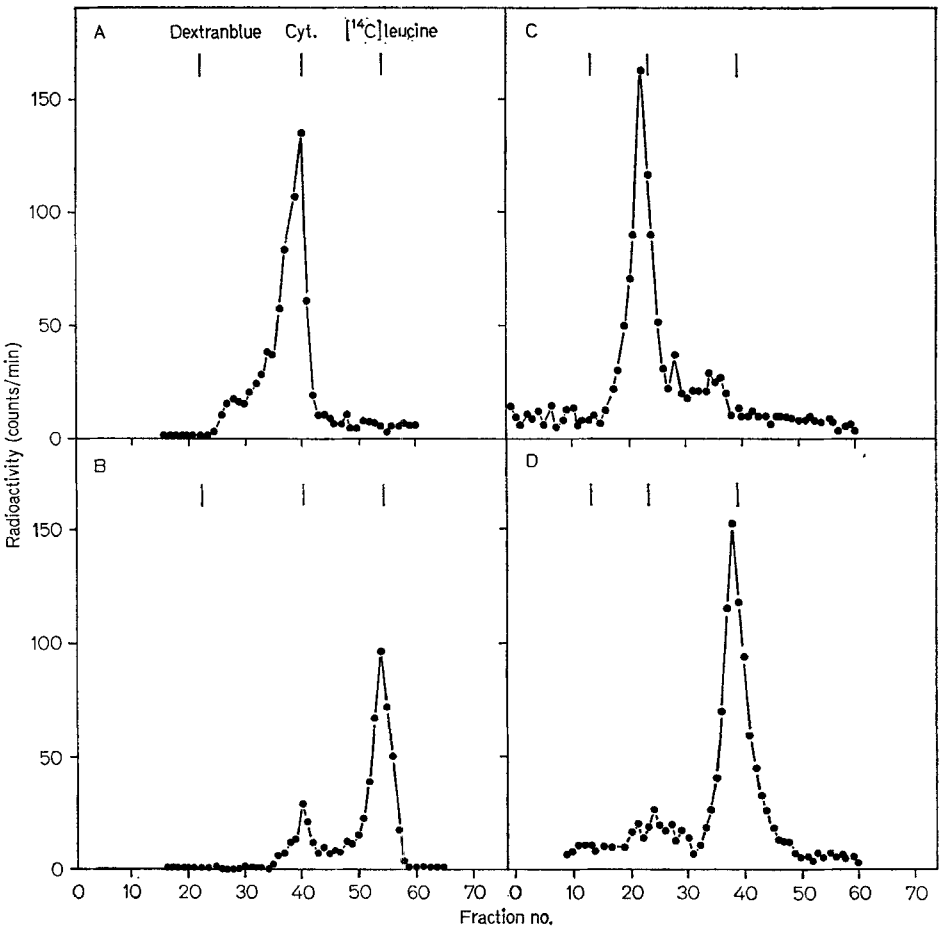


Fig. 7. *Gel chromatography on Sephadex G-200 of radioactively labeled nascent polypeptide chains associated with mitochondrial monomeric and polymeric ribosomes, before and after treatment with trypsin.* Cyt: cytochrome *c*. (A) Monomeric ribosomes; (B) monomeric ribosomes, treated with trypsin; (C) polymeric ribosomes and (D) polymeric ribosomes, treated with trypsin

Treatment of polymeric ribosomes with ribonuclease brings about a shift of part of the radioactivity towards lower apparent molecular weights (Fig. 5D). Still no distinct peaks are visible. In the case of monomeric ribosomes, the first peak on the gel disappears completely and all radioactivity is found in the second one (Fig. 5B). This suggests that the peak with the apparent molecular weight of 27000 represents peptidyl transfer RNA. The lability of the material appearing in this peak in media with alkaline pH supports this conclusion. Free transfer RNA (phenylalanine-specific from yeast) migrates on the gel to a position corresponding to an apparent molecular weight of 15000.

In Fig. 6, gel-electrophoretic analysis of nascent peptide chains on cytoplasmic ribosomes is shown. With both, monomeric and polymeric ribosomes a broad distribution of radioactivity is observed. No significant differences between monomeric and polymeric forms can be detected (Fig. 6A, C). Treatment of ribosomes with ribonuclease, in both cases partly shifts the radioactivity to lower apparent molecular weight, indicating the presence of peptidyl transfer-RNA. Furthermore, no significant difference between monomeric and polymeric ribosomes in the distribution of labeled chains on the gels can be detected after ribonuclease treatment (Fig. 6B, D).

Analysis of Nascent Peptide Chains Associated with Mitochondrial Ribosomes by Gel Chromatography

In a second approach to characterize labeled nascent peptide chains, the mitochondrial ribosomes were dissolved in dodecylsulfate-containing buffer and then incubated with ribonuclease. They were applied on Sephadex G-200 columns, equilibrated with dodecylsulfate buffer. Monomeric as well as polymeric ribosomes display a single elution peak of radioactivity (Fig. 7A, C). In both cases the labeled peptides are eluted together with the marker cytochrome *c*. They are clearly separated from low-molecular-weight components such as

leucine, which as ^{14}C-labeled compound was run together with the ^{3}H-labeled peptides.

If dodecylsulfate was omitted from the elution buffer and from the equilibration buffer, the radioactive peptides could not be eluted from the column. The radioactivity stayed fixed to the gel at the top of the column.

When the ribosomes were incubated with high concentrations of trypsin (1% final concn) for long periods (6 h), the nascent peptides associated with monomeric as well as polymeric ribosomes, were digested. This is demonstrated in Fig. 7B, D. After digestion, the bulk of the radioactivity is eluted together with free leucine.

Chromatography on Biogel P-30 under the same conditions also gives molecular weights of 11000 for the undigested translation products. In this system, the digested products are eluted before the marker leucine.

Appearance of Mitochondrial Translation Products in the Mitochondrial Membrane

In order to study the appearance of the polypeptides synthesized at the mitochondrial ribosomes in the mitochondrial membrane, two different sets of experiments were carried out.

In a first experiment, cells were exposed to cycloheximide and then a 1.5-min pulse of [^{3}H]leucine was given. One portion of cells was immediatly withdrawn and cooled to 0 °C. A chase of unlabeled leucine was then added to the hyphal suspension. Equal portions were now removed and cooled, after 15, 30 and 60 min, respectively. Mitochondrial membranes were isolated from all portions. The specific radioactivities of the total mitochondrial-membrane protein were the same in all samples. This demonstrates the effectiveness of the chase, taking into account that the cells do not grow in the presence of cycloheximide.

Fig. 8 presents the results of the gel-electrophoretic separation of the membrane preparations. In membranes from cells exposed to a 1.5-min pulse without a subsequent chase, radioactivity was mainly found in two peaks corresponding to molecular weights of 18000 and 11000. A smaller part of the radioactivity appears in the higher-apparent-molecular-weight region between 20000 and 40000 (Fig. 8A). During the chase period, the proportion of the total radioactivity in the two smaller bands is diminished, especially that of the 11000-mol.wt band. The bands with apparent molecular weights between 20000 and 40000 increase strongly (Fig. 8B, C, D).

Pulse-chase experiments with *Neurospora* cells in the presence of cycloheximide demonstrate that a 30-s chase period is enough to remove 80% of the radioactivity associated with the mitochondrial

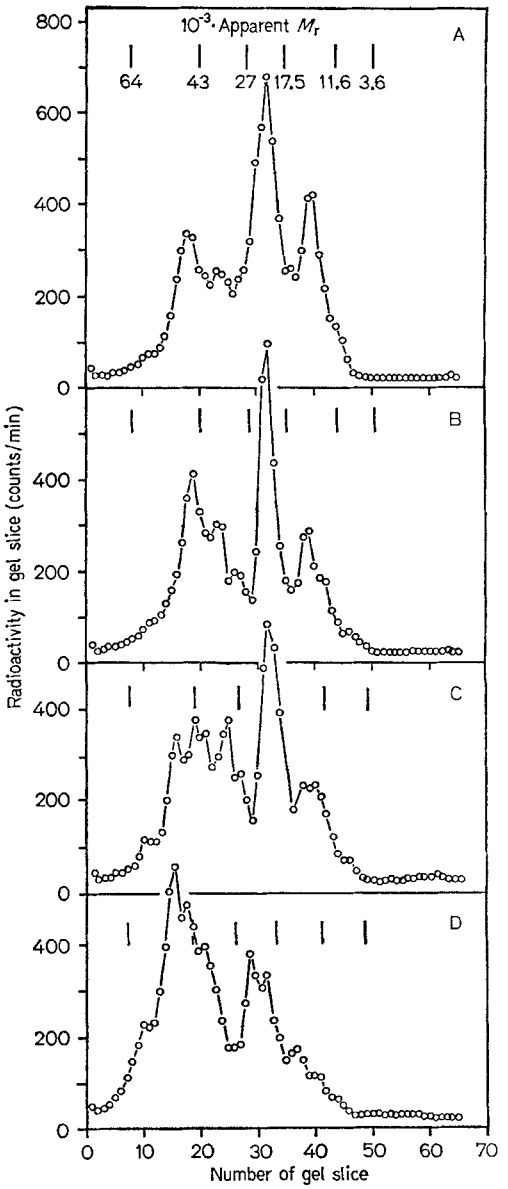


Fig. 8. *Gel-electrophoretic analysis of mitochondrial membranes after pulse-chase labeling with [^{3}H]leucine* in vivo *in the presence of cycloheximide.* A constant pulse period (1.5 min) was followed by different chase periods. (A) Without chase; (B) 5-min chase; (C) 15-min chase and (D) 60-min chase

ribosomes. Obviously, the components with lower apparent molecular weights, observed after short time labeling in the membrane, do not represent unfinished peptide chains associated with or released from the mitochondrial ribosomes.

In a second experimental set-up, isolated mitochondria were incubated with [^{3}H]leucine, in a medium optimal for amino acid incorporation, for different lenghts of time. Again the mitochondrial

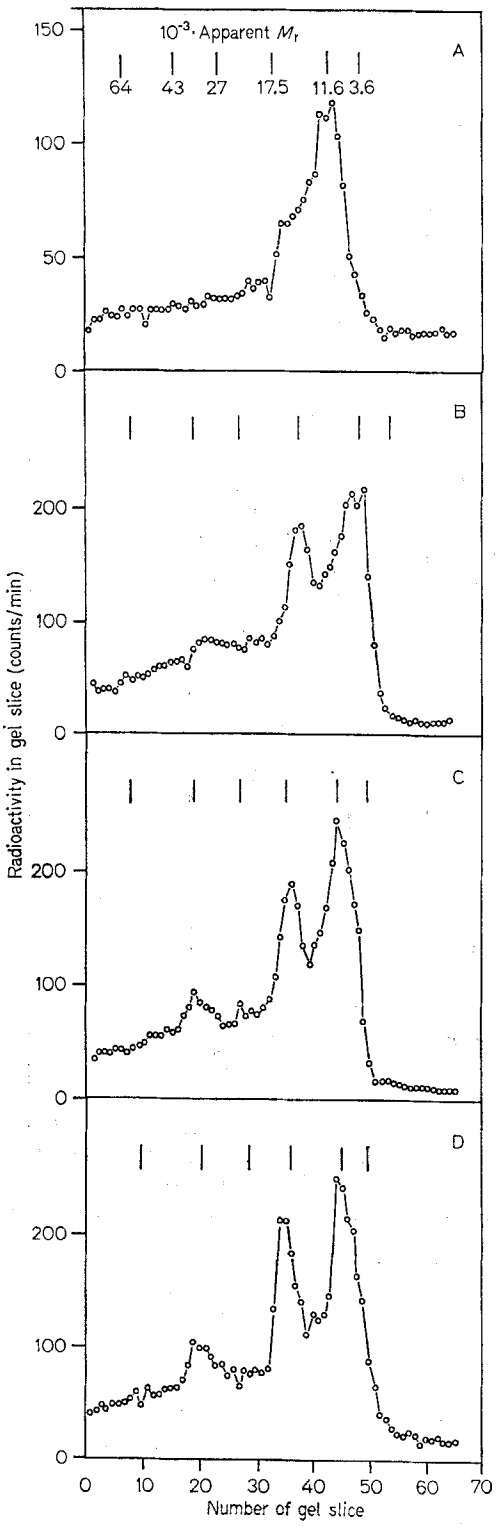


Fig. 9. *Gel-electrophoretic analysis of mitochondrial membranes after labeling* in vitro *with [^{3}H]leucine.* Labeling periods: (A) 5 min; (B) 15 min; (C) 30 min and (D) 60 min

membranes were isolated and subjected to gel electrophoresis. The result is shown in Fig. 9. After short (5 min) and long incorporation periods (60 min) two peaks with apparent molecular weights of 18000 and 11000 are prominent in the radioactivity patterns of the gels. In contrast to the situation *in vivo*, radioactivity in the region corresponding to apparent molecular weights of 20000 to 40000 is quite low after prolonged incorporation *in vitro*.

DISCUSSION

Translation Products on Mitochondrial Ribosomes

The polypeptides labeled by the procedure described in this paper really are mitochondrial translation products, as proven by the following characteristics: (a) they can be completely released from the ribosomes by puromycin and (b) after the disintegration of the ribosomes they can be recovered as peptidyl transfer RNA.

These polypeptides appear to have some peculiar properties. The most interesting one is the extreme tendency to aggregate. Either as free peptides or in the form of peptidyl transfer RNA and peptidyl puromycin, they can only be kept in solution if detergents such as sodium dodecylsulfate are present. In contrast, the nascent peptide chains of cytoplasmic ribosomes, or at least the bulk of them, are water-soluble compounds.

Furthermore, mitochondrial ribosomes in contrast to cytoplasmic ribosomes also show a high tendency to aggregate. The distribution of radioactivity in the density gradients indicates that it is especially those ribosomes which carry nascent peptide chains which aggregate. When these chains are removed with puromycin, the amount of ribosomes in the gradient pellet is greatly reduced. This means that the tendency to aggregate is at least partly conferred by the nascent polypeptide chains.

This peculiarity of mitochondrial ribosomes is pertinent to the question of whether the polymeric ribosomes observed in the density gradients are real complexes of messenger RNA and ribosome or merely aggregates of monomeric ribosomes. The following observation has to be considered in this respect: cytoplasmic ribosomes can be easily converted to monomeric ribosomes by treatment with low concentrations of ribonuclease. In contrast, mitochondrial polymeric ribosomes are not destroyed by comparatively excessive amounts of pancreatic or T_1 ribonuclease. Unless we assume a quite uncommon specific stability of mitochondrial messenger RNA or the mitochondrial messenger RNA · ribosome complex, this would mean that the polymeric ribosomes are aggregates. The aggregates may be formed from monomeric ribosomes or functional messenger-bound polyribosomes can be

artifactually interconnected. With the mitochondrial ribosomes from HeLa cells a similar behaviour has been described, and it was concluded that the polymeric forms are aggregates stabilized by nascent peptide chains [21].

There is no definite proof for the existence of functional complexes of polyribosomes and messenger RNA in mitochondria. Arguments in this direction have been brought up for *Euglena* [23]. In electron micrographs of yeast mitochondria, structures which might represent such complexes have been visualized [24]. So it may well be that they exist but are destroyed during isolation of the ribosomes.

It is hereby relevant to characterize the nascent peptide chains adhering to monomeric and polymeric ribosomes. In the case of cytoplasmic ribosomes, electrophoresis on dodecylsulfate gels reveals no significant differences in the apparent size distribution of the nascent peptide chains on monomeric and polymeric ribosomes. This is in accordance with the observation, that monomeric ribosomes can be made from polymeric ribosomes by the action of ribonuclease, and with the assumption that the monomeric ribosomes present in sucrose-density gradients are derived from polymeric ribosomes by degradation during isolation.

In the case of mitochondrial ribosomes however, a clear difference in the electrophoretic properties of peptide chains adhering to polymeric and monomeric ribosomes is found. Whereas the translation products at the polymeric ribosomes display a broad distribution of their apparent molecular weights after dodecylsulfate-gel electrophoresis, the product associated with the monomers appears to be quite uniform. Before ribonuclease treatment this product shows a distinct peak with an apparent molecular weight of 27000, probably representing peptidyl transfer RNA. After ribonuclease treatment or after incubation at alkaline pH, a double peak in the apparent-molecular-weight region of 5000—10000 is obtained. The electrophoretic procedure used in our experiments does not allow a more accurate determination of the apparent molecular weight in this region. Furthermore it does not allow an opinion on the number of individual translation products.

In order to explain the difference between the translation products on mitochondrial polymeric and monomeric ribosomes, we essentially have to regard the following possibilities:

a) The monomeric forms of mitochondrial ribosomes separated by gradient centrifugation carry complete polypeptide chains as peptidyl transfer RNA. The three-dimensional structure of these ribosome · product complexes does not allow them to form aggregates. In contrast, the polymeric ribosomes (and the ribosomes in the gradient pellets) carry unfinished chains. In the intact cell, as real messenger RNA · polyribosome complexes they cannot aggregate, however during isolation this artifact can occur. Actually, if isolated mitochondrial ribosomes are kept at 37 °C a further and preferential aggregation of the polymeric ribosomes takes place.

b) The basis of the second possible explanation is to assume artifacts. For instance, mitochondria might contain a proteolytic enzyme which specifically attacks the nascent peptide chains at the ribosomes and breaks them down to a certain length. The residual chain would be that part which is embedded in the ribosomal structure. This hypothetical process would produce the monomeric ribosomes, whereas on the polymeric ones still-projecting chains are present, which lead to aggregation.

There are some objections against the second interpretation. Gel filtration does not show those differences in the apparent molecular weights of chains associated with monomeric and polymeric ribosomes, as displayed by gel electrophoresis. The molecular weights evaluated by this latter technique have to be considered with great care. The linear dependency of the electrophoretic mobility from log (molecular weight) [25] does not generally hold for smaller peptides [26,27]. On our gels, the major part of free leucine remains at the origin and a small part migrates into the gel. Moreover, it is not excluded that extremely hydrophobic peptides form aggregates even in the presence of dodecylsulfate.

A central part of the first hypothesis is the assumption that part of the mitochondrial ribosomes carry complete polypeptide chains. In this regard the following observations are important:

a) In the case of cytoplasmic ribosomes, the specific radioactivity of the polymeric and monomeric ribosomes in the gradient is the same. In contrast, with mitochondrial ribosomes a much higher specific radioactivity is observed in polymeric ribosomes. This could either mean that there is a pool of mitochondrial ribosomes inactive in protein synthesis under the conditions of the experiment. It could as well mean that there is a pool of monomeric ribosomes which carry completed polypeptide chains. The pool might only be slowly diluted by pulse-labeled monoribosomes. Pulse-chase experiments however, do not show a transition of the label from the polymeric into the monomeric ribosomes (unpublished results). Hence, it is not probable that the hypothetic pool of monoribosomes carrying completed chains, is large compared to the pool of ribosomes active in chain elongation. A large part of the monoribosomes and of the free subunits may therefore represent ribosomes which have delivered the polypeptide to the membrane and have not reinitiated.

b) Our experimental conditions do not guarantee an abrupt stop of the synthetic process. Although

the incorporation period is stopped by rapid cooling of the cells to 0 °C, this does not necessarily mean that polypeptide synthesis is immediatly ended. With bacterial systems it has been shown that chain elongation can proceed below 8 °C, whereas initiation is blocked [28]. It usually takes 1 h, until mitochondria are isolated from *Neurospora* cells and can be lysed for the preparation of ribosomes. During this period there would be ample time for the elongation of initiated chains. Part of the chains might be completed but not delivered to the membrane. Such a mechanism could explain the astonishing observation that upon gel chromatography of the chains associated with polymeric and monomeric ribosomes practically no low-molecular-weight components are seen. Only after tryptic digestion, low-molecular-weight components appear.

Translation Products in the Mitochondrial Membrane

The first hypothesis implicates that the translation products have apparent molecular weights of about 11000 according to gel filtration and of 5000—10000 according to gel electrophoresis. At first sight this is difficult to reconcile with the following observation: after gel electrophoresis of mitochondrial membranes, bands are found with apparent molecular weights up to 40000 which are labeled with radioactive amino acids in the presence of cycloheximide [17,29—33]. However, kinetic studies presented in this paper indicate that, at least, the bands with apparent molecular weights in the region of 20000 to 40000 are not original translation products, but are rather formed from material with a lower apparent molecular weight. It seems that the original translation products are smaller components and that a modification process takes place in the membrane. This process is slow compared to the translation process, at least under the conditions of the experiment. The nature of this modification is not known. Speculations lead towards specific aggregation reactions as well as chemical modification reactions. The inability to observe this process *in vitro*, would suggest that it is hindered when the mitochondrion is not in its natural surrounding.

In a recent publication, Tzagoloff and Akai [31] have suggested that in yeast the major product of mitochondrial protein synthesis is a protein with an apparent molecular weight of 7800 (gel electrophoresis). These authors claim, that in the high-molecular-weight components, this protein is either present in a polymeric form or associated with some other components. At present it is not possible to decide whether there is any connection between these and our findings.

Some considerations about the mechanism by which the mitochondrial translation products find their way from the ribosome to their functional site in the membrane may be based on the results presented in this paper. Since the translation products show a very strong tendency to aggregate, it is difficult to imagine that they migrate freely through the matrix. Essentially two possible mechanisms have to be discussed with regard to this integration process:

a) The monomeric ribosomes transport the complete peptide chains in the form of peptidyl transfer RNA to the membrane. After cleavage of the ester bond, the peptide is inserted into the membrane and the ribosome returns into the ribosomal cycle.

b) Mitochondrial ribosomes are bound to the inner membrane and insert the growing peptide chain directly into the membrane.

To decide between these alternatives, it is necessary to know the exact localisation of the ribosomes inside the mitochondrion. On the basis of indirect arguments, it has been suggested that the ribosomes are part of the inner mitochondrial membrane [34]. However, in a variety of organisms, mitochondrial ribosomes have been visualized by electron microscopy in the matrix, more or less close to the inner membrane [35]. These observations do not prove or exclude that at least part of them is bound to the inner membrane. Data presented in this report indicate that if ribosomes carrying nascent peptide chains are attached to the inner membrane, this does not occur in the same strong way as with ribosomes that are attached to the membranes of rat-liver endoplasmic reticulum. It is quite possible that they undergo a transient attachment to the membrane. We accept the first of the two mentioned mechanisms as a working hypothesis.

The extremely hydrophobic character of the mitochondrial translation products may have relevance for speculations on the existence of the mitochondrial protein synthesis. It seems possible that the hydrophobic properties make it necessary that these peptides are delivered to the inner membrane from the matrix side, since they cannot be transported through the cytoplasm and the intercristae space. Thus, on the basis of the endosymbiont theory of the evolution of mitochondria, it seems reasonable that DNA and a special system of transcription and translation had to be maintained to form a few peptides for a functional inner membrane, in the compartment enclosed by this membrane.

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