MEMBRANE-ASSOCIATED RIBOSOMES IN
MITOCHONDRIA OF *NEUROSPORA CRASSA*

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INTRODUCTION

The *poky* (mi-1) strain of *Neurospora crassa* is a useful test organism in which to evaluate methods for isolating mitochondrial ribosomes actively engaged in protein synthesis. In *poky* mitochondria there is a deficiency of small ribosomal subunits and the mass ratio of large to small subunit RNA is ~10:1 (1). Nonetheless, mitochondrial protein synthesis can be observed in *poky* (2) and cytochrome oxidase which is in part synthesized by *Neurospora* mitochondria (3, 4) is present at a level

~30% of wild type activity (5). The mitochondrial polysomes active in this synthesis can be expected to have a ribosomal subunit mass ratio (large/small) of 2:1. Polysome preparations with higher subunit ratios can be considered to be contaminated by free large subunits.

Until now all preparations of mitochondrial polysomes have been obtained from organelles lysed by detergents. The polysomes are collected directly as a rapidly sedimenting fraction in analytical sucrose gradients (6–9) or as a pellet sedimenting through high molarity sucrose in a step gradient (8). In applying these methods to *poky* mitochondria and analyzing the subunit content of the polysome preparations after incubation with puromycin, we found that mass ratios of large to small ribosomal subunits were not 2:1 but were close to 10:1. For this reason we have explored new methods of obtaining active mitochondrial ribosomes, taking advantage of the recent report by Chua et al. (10) that in the chloroplast of *Chlamydomonas* ribosomes active in protein synthesis are associated with thylakoid membranes.

In this paper we report that when mitochondria of *N. crassa* are disrupted by sonication the majority of ribosomes sediment with the membrane vesicle fraction. Most of these can be released as subunits by incubation with puromycin and high salt. In *poky* the mass ratio of the released subunits (large/small) is ~2:1. Vesicles retain subunits, recoverable after detergent lysis, in a mass ratio of ~10:1. Ribosomes releasable by puromycin-high salt treatment and those retained in the vesicle fraction appear to have morphological counterparts in electron micrographs of thin sections of the preparations. The former appear to be ribosomal particles occurring in clusters or linear arrays on the outer surfaces of vesicles formed from inner membranes with the matrix side out; the latter appear to be ribosomes trapped within partially disrupted mitochondria.

MATERIALS AND METHODS

Culture Conditions

Experiments were carried out with *me-3* (designated M-3) and *poky* F⁺ *me-3* (designated *poky*), methionine requiring strains of *N. crassa*. These strains were chosen because they were under intense study for another project in our laboratory. The methionine requiring *poky* strain was derived from a cross of *poky* F⁺ with *me-3*. Both the *poky* F⁺ *me-3* and

me-3 strains were backcrossed three times with wild type RL-38A to obtain the organisms actually used. Large stocks of lyophilized conidia were prepared and used at 2-wk intervals to initiate new vegetative cultures.

Methods for the growth of *Neurospora* in liquid culture are those described previously (11) except that culture media were supplemented with L-methionine (1.5 mM). Mycelia were harvested in log growth phase after 14 h (M-3) or 24 h (*poky*) growth. Cultures were started with an inoculum of 7 × 10⁵ conidia/ml and maintained at 25°C. In some experiments, chloramphenicol suspended in sterilized H₂O was added to a final concentration of 3 mg/ml 1 h before harvesting. Disruption of mycelia and preparations of crude mitochondria were carried out as described previously (11).

Purification and Disruption of Mitochondria

Purification of mitochondria from crude preparations was carried out by flotation in sucrose gradients (44–54%) as described previously (12). We have demonstrated that these mitochondria are free of contamination by ribosomal RNA of cytoplasmic origin (12). Purified mitochondria were suspended (20–30 mg protein/ml) in 25 mM KCl, 25 mM MgCl₂, 25 mM Tris HCl pH 7.5, 5 mM dithiothreitol (DTT) (LKMTD), sonicated at 0°C for 20 s in the Branson sonifier (Branson Instruments Co., Stamford, Conn.) at 6–7 A, and cooled for 1 min. The sonication procedure was repeated three more times, and the suspension was centrifuged at 25,000 g for 10 min to obtain mitochondrial supernatant (S25) and membrane fractions (P25). In order to remove any free ribosomes that might have been trapped in the P25 fractions, they were suspended in LKMTD with a loose-fitting Potter homogenizer and the suspension centrifuged again at 25,000 g for 10 min. The supernate was combined with S25 and used for further analysis.

High Salt-Puromycin Treatment of P25 and S25 Fractions

P25 fractions were treated with high salt and puromycin as described by Blobel and Sabatini (13) by suspension in 5 ml of 500 mM KCl, 25 mM Tris HCl pH 7.5, 5 mM DTT, 10 mM MgCl₂ or 25 mM MgCl₂ (HKMTD₁₀ or HKMTD₂₅). The KCl concentration of the combined S25 was adjusted to 500 mM by adding solid KCl. After addition of 0.1 volume of 10 mM puromycin (adjusted to pH 7.6 with KOH), the reaction mixtures were incubated at 35°C for 10 min and then centrifuged at 25,000 g for 10 min to remove membrane vesicles.

Preparation of Mitochondrial Polysomes

Mitochondrial polysomes were prepared by the method of Nist (8) modified in the following way. Purified mitochondria were suspended in LKMTD and lysed by adding Nonidet P40 to a concentration of 1%. The lysate was layered over 7 ml of 1.8 M sucrose in LKMTD and centrifuged at 55,000 rpm in an A321 rotor of the International centrifuge (International Equipment Co., Needham Heights, Mass.), model B60, for 18 h. The polysomal pellet was suspended in $HKMTD_{25}$ and incubated with puromycin at 35°C for 10 min. Aliquots of the reaction mixtures were layered onto 5-20% analytical sucrose gradients. The gradients were centrifuged at 39,000 rpm for 3 h at 18°C in a SB 283 rotor of the IEC centrifuge and analyzed by recording the absorbancy at 254 nm using an ISCO (Instrumentation Specialties Co., Lincoln, Nebr.) gradient fractionator.

Electron Microscopy

Mitochondria and P25 fractions were pelleted and then fixed in 0.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 containing 7% sucrose and 25 mM $MgCl_2$ for 30 min at 0°C followed by 1% osmium in the same buffer at 0°C overnight. The specimens were stained in uranyl acetate and then embedded in Epon. Sections, doubly stained with uranyl acetate and lead, were examined in a Siemens Elmiskop 1B electron microscope.

Chemicals

Chloramphenicol and puromycin were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Nutritional Biochemical Corp., (Cleveland, Ohio), respectively. Sucrose (ribonuclease-free) was obtained from Schwarz/Mann (Div., Becton, Dickinson & Co., Orangeburg, N. Y.). Nonidet P40 was a gift from Shell Chemical Corp., New York. Other chemicals were reagent grade.

RESULTS

The Distribution of Ribosomal Subunits in Subfractions of M-3 Mitochondria

Mitochondria disrupted by sonication were subfractionated into S25 and P25 fractions by centrifugation. The vesicle fraction was then treated with puromycin in the presence of high salt to obtain released subunits, after which a final vesicle pellet was collected by centrifugation. The subunit content of this pellet was determined after detergent lysis. In each of the three fractions the ribosomal subunit content was analyzed by the sucrose gradient centrifugation methods de-

scribed in Fig. 1. Based on the sum of OD_{254} recovered in the three fractions, the percentage distribution of ribosomal subunits shown in Table I was calculated.

In M-3 mitochondria the mass ratio of large to small ribosomal subunits is 2:1 for total mitochondrial extracts and for all of the subfractions shown in Table I. The data shown represent the summed large and small subunit OD_{254}. As indicated in Table I the major fraction ($\sim\frac{2}{3}$) of ribosomal subunits sediment with mitochondrial vesicles and more than half of these are released by incubation with puromycin. About 25% of the total ribosome subunit population remains with the vesicle fraction and can be released by detergent lysis.

It was observed by Chua et al. (10) that pretreatment of Chlamydomonas cultures with chloramphenicol, strikingly increased the fraction of chloroplast ribosomes associated with thylakoid membranes. This effect was interpreted as an inhibition of spontaneous peptide termination by the antibiotic acting selectively on organelle ribosomes, which helped to preserve the polysome-membrane association.

As shown in Table I we studied the effect on ribosomal subunit distribution of addition of chloramphenicol to cultures 1 h before harvesting. We consistently found that antibiotic pretreatment reduced the ribosome content of S25, increased the content of subunits releasable from P25, and to a small extent increased the subunit content of the final pellet. These results are quantitatively less striking than those in the chloroplast of Chlamydomonas (10). The difference in behavior of the two organisms may reflect the fact that preparation times for Neurospora are shorter and the chance for spontaneous peptide termination lower. But the reproducible chloramphenicol effect on the content of mitochondrial ribosomal subunits in P25 suggests that ribosomes associated with mitochondrial membranes which are released by puromycin-high salt treatment may be active in protein synthesis.

Membrane-Bound Ribosomes in the Poky Strain

Several attempts were made to prepare polysomes from detergent lysed poky mitochondria in analytical sucrose gradients and in preparative step gradients. In each case, when the ribosomal

Polysomes	P 25	S 25

(M-3 Mitochondria)

a   b   c

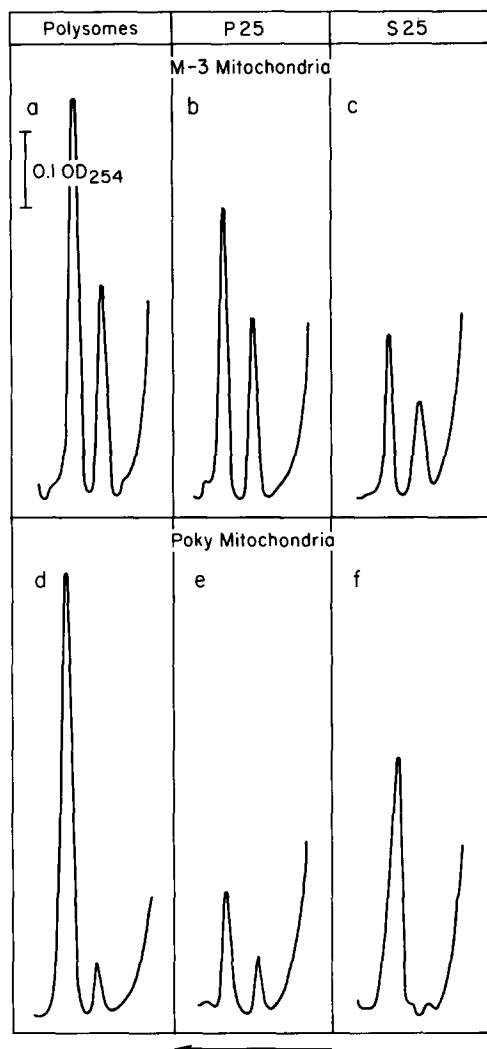Poky Mitochondria

d   e   f

0.1 OD$_{254}$

FIGURE 1 Sucrose density gradient analysis of ribosomal subunits present in S25, P25, and in polysome preparations of M-3 and *poky* cultures. Purified mitochondria (20-culture flasks) from chloramphenicol-treated cells were suspended in 10 ml of LKMD. One-half of the suspension was sonicated in a Branson sonifier and then centrifuged at 25,000 g for 10 min to obtain S25 and P25. The concentration of KCl in S25 was adjusted to 0.5 M by adding solid KCl. After washing with LKMTD, P25 was suspended in 5 ml of HKMTD$_{10}$. The other half of mitochondrial suspension was lysed in 1% Nonidet P40 and used for the preparation of a polysome pellet (Materials and Methods). The polysome pellet was suspended in 5 ml of HKMTD$_{10}$. Solutions of S25, P25, and polysome pellets were treated with 1 mM puromycin at 35°C for 10 min. Then the reaction mixture of P25 was centrifuged again at 25,000 g for 10 min to remove membrane vesicles. Equivalent samples of these reaction mixtures

subunit content was determined after puromycin treatment, the mass ratio of large to small subunits approached 10:1. A typical result for polysomes prepared by a step gradient method is shown in Fig. 1 d. The comparable experiment with control (M-3) mitochondria is shown in Fig. 1 a. The subunit ratio in mitochondrial polysomes from *poky* is ~10:1 and this ratio suggests that excess large subunits are entrapped nonspecifically.

Aliquots of the same mitochondrial suspensions used for the polysome preparations were sonicated to prepare P25 and S25 fractions. The P25 fraction was treated with puromycin-high salt to release ribosomal subunits. The subunit content was analyzed by sucrose gradient analysis as shown in Fig. 1. It is significant that ribosomal subunits released from the P25 vesicles of *poky* mitochondria by puromycin-high salt treatment have a mass ratio (large/small) of 2:1 (Fig. 1 e) while those in S25 are present in a ratio of >50:1 (Fig. 1 f).

This type of experiment was repeated to determine the subunit content of the final membrane pellet remaining after puromycin treatment of P25 and to quantitate the distribution of large and small subunits among the three subfractions. The results are shown in Table I for untreated *poky* cultures and for cultures incubated with chloramphenicol. In both cases the majority of small subunits sediment with the vesicle fraction, and chloramphenicol pretreatment increases the small subunit content of the puromycin releasable-P25 fraction, apparently at the expense of the S25 fraction. The change is strikingly reflected in the large/small subunit mass ratios of S25 from chloramphenicol-treated and untreated cultures. In both cases however, the ratio of subunits released by puromycin from P25 is ~2:1, the mass ratio expected for polysomes; and the ratio of subunits remaining in the final pellet is ~10:1, the ratio of subunits present in total mitochondria as determined by the mitochondrial ribosomal RNA content (1).

(0.3–0.5 ml) were layered onto 5–20% sucrose in HKMTD$_{25}$ and centrifuged at 39,000 rpm for 3 h in a SB 283 rotor of the IEC centrifuge. The gradients were analyzed by monitoring OD$_{254}$ with an ISCO gradient fractionator. The same protocol was followed with *poky* mitochondria except that volumes of the total ribosome suspension and S25 were 1.5-fold that of P25. The arrow at the bottom of the figure designates the direction of the centrifugal field.

TABLE I

Ribosomal Subunit Content of Fractions Derived from Mitochondria of M-3 and Poky Strains Treated
With or Without Chloramphenicol

| | M-3 | | Poky | | | | | | |
| | | | -CAP | | | +CAP | | | |
	-CAP	+CAP	L	S	L/S		L	S	L/S
	%	%	%	%			%	%	
Supernate (S25)	38	23	54	22	25:1		50	10	50:1
Released from P25 by high salt + PM	37	48	8	42	2:1		11	54	2:1
Released from the final pellet by Nonidet	25	28	38	36	10:1		39	35	11:1

Experiments were carried out as described in the text and Materials and Methods. +CAP: cells were treated with chloramphenicol for 1 h before harvest. P25 was treated with high salt and puromycin and then centrifuged at 25,000 rpm for 10 min. The supernate was analyzed for its subunit content and the final membrane pellet was lysed in 1% Nonidet P40. The lysates were layered over 2.5 ml 1.8 M sucrose in LKMTD and centrifuged at 55,000 rpm for 18 h to obtain a ribosome pellet. The ribosome pellet was processed as described in Fig. 1 for analysis of subunits.

Distribution (%) was obtained from OD_{254} profiles of ribosomal subunits on 5-20% sucrose gradients, and is expressed at percent total OD_{254} recovered in the three fractions. Values for M-3 include both large and small subunits and for poky, large (L) and small (S) subunit data are given separately. The OD_{254} ratio for large/small subunits (L/S) is given for poky mitochondrial fractions. The data are taken from a single representative experiment.

Dissociation of Ribosomes Bound to Mitochondrial Membranes

In interpreting the puromycin-high salt effect on the endoplasmic reticulum of hepatocytes, Adelman and his co-workers (14) have emphasized two factors in the association of ribosomes with membranes: salt-sensitive bonds and attachment through the nascent peptide chain. Disruption of both bonds, the first by exposure to high salt and the second by chain termination with puromycin, is required for release.

Experiments were undertaken to reveal the requirement for simultaneous disruption of these bonds to completely release ribosomes from their association with mitochondrial membranes. Release from P25 was measured in the presence of low (25 mM) and high (500 mM) KCl concentrations and the results for poky and M-3 mitochondria are shown in Fig. 2. Quantitative data are given in Table II. From these data it can be concluded that like other ribosome membrane systems (10, 13, 34), maximum release requires treatment with high salt and puromycin, the findings that high salt alone (Fig. 2 b and 2 e and Table II) causes ~50% release from P25 and that puromycin-low salt treatment releases only 10–30% (Fig. 3 c and

3 f and Table II) are similar to those obtained with hepatocyte endoplasmic reticulum membranes (13, 14) and chloroplast membranes (10).

In other experiments we also observed a critical role of $MgCl_2$ concentration in influencing subunit release. At 10 mM $MgCl_2$, 500 mM KCl, puromycin gave more efficient subunit release from membranes than under the same conditions with 25 mM $MgCl_2$. However, at lower $MgCl_2$ concentrations (less than 5 mM) and especially in low KCl, the centrifugation patterns indicated that disruption and alteration of sedimentation behavior of released small subunits had taken place.

Electron Microscope Studies

Pellets of P25 fractions, and puromycin-high salt-treated P25 fractions were fixed, cut into 1 mm slices, and embedded in Epon. Thin sections of these oriented blocks were examined in the electron microscope. Representative views of the upper portion of the pellets are shown in Fig. 3. Before puromycin treatment the P25 fraction consists of single-walled membrane vesicles of varying size, as shown in Fig. 3 a, vesicles within vesicles, and mitochondria which are only partially

fragmented. The latter two components are especially prominent in the lower portions of the pellet. Vesicles sectioned through their centers are devoid of content. Grazing sections of the vesicles
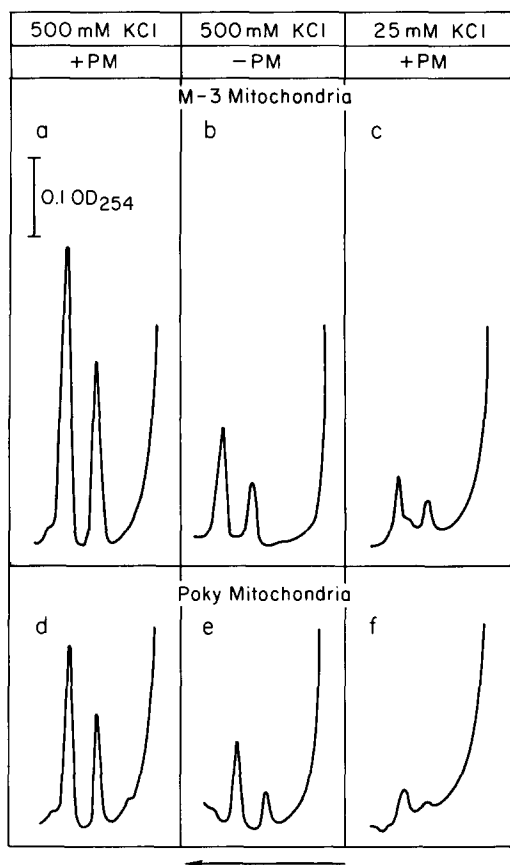

FIGURE 2 Effect of KCl concentrations and puromycin on the release of ribosomes from P25 of M-3 and *poky* mycelia. P25 was prepared from chloromphenicol-treated M-3 or *poky* cultures as described in Fig. 1 and the Materials and Methods. The high salt (500 mM KCl) treatment of the P25 fraction with or without puromycin was carried out in HKMTD$_{10}$ as described in Fig. 1 and the Materials and Methods. The low salt (25 mM KCl) reactions were carried out similarly in LKMTD. The reaction mixtures (about 15 and 25 mg mg protein/ml of P25 from M-3 and *poky* mitochondria) were centrifuged at 25,000 g for 10 min and aliquots of the supernate from each reaction mixture were layered onto 5–20% linear sucrose gradients in HKMTD$_{25}$. After centrifugation at 39,000 rpm for 3 h at 18°C in a SB 283 rotor of the IEC centrifuge, the gradients were analyzed as described in Fig. 1. The arrow at the bottom of the figure indicates the direction of the centrifugal field.

TABLE II

Relative Amounts of Ribosomes Released from P25, Obtained from Chloramphenicol-Treated M-3 and Poky Mycelia, Upon Incubation With High and Low KCl Concentrations in the Absence or Presence of Puromycin

	M-3	*Poky*
500 mM KCl + PM	100	100
− PM	42	50
25 mM KCl + PM	29	25
− PM	10	15

Experiments were carried out as described in Fig. 2. The relative amounts of ribosomes released from mitochondrial membrane fractions (P25) were calculated from the ribosomal subunit profiles at 254 nm. Values are expressed as per cent release, taking as 100 the subunit release obtained by combined high salt and puromycin treatment. The numbers are averages of three experiments.

contain electron-dense ribosomal particles arranged linearly or in small clusters. Careful inspection of such tangential views indicates that these ribosomes are attached to the outside surface of most vesicles. Since in intact mitochondria we do not observe ribosomes in the intracristal space, these microscope findings in P25 suggest that after sonication membrane vesicles derived from the inner membrane form with the matrix surface facing out. A similar interpretation for the formation of sonication-submitochondrial vesicles has been made on the basis of the distribution of inner membrane particles which are visible with negative staining (15).

After treatment with puromycin and high salt the most striking change in P25 morphology is the extensive depletion of ribosomal particles. When careful search is made for ribosomes still present they are usually found within complex double-walled vesicles and most often within partially fragmented mitochondria which are mainly present in the bottom of the P25 pellet.

Comparable studies with P25 derived from *poky* mitochondria have been carried out. The findings are similar to those in M-3 mitochondria but the content of ribosomes, especially those visible on surfaces of vesicles, is markedly reduced.

DISCUSSION

Mitochondria which have been sonicated can be fractionated into supernatant and vesicle subfractions by centrifugation. The supernate contains
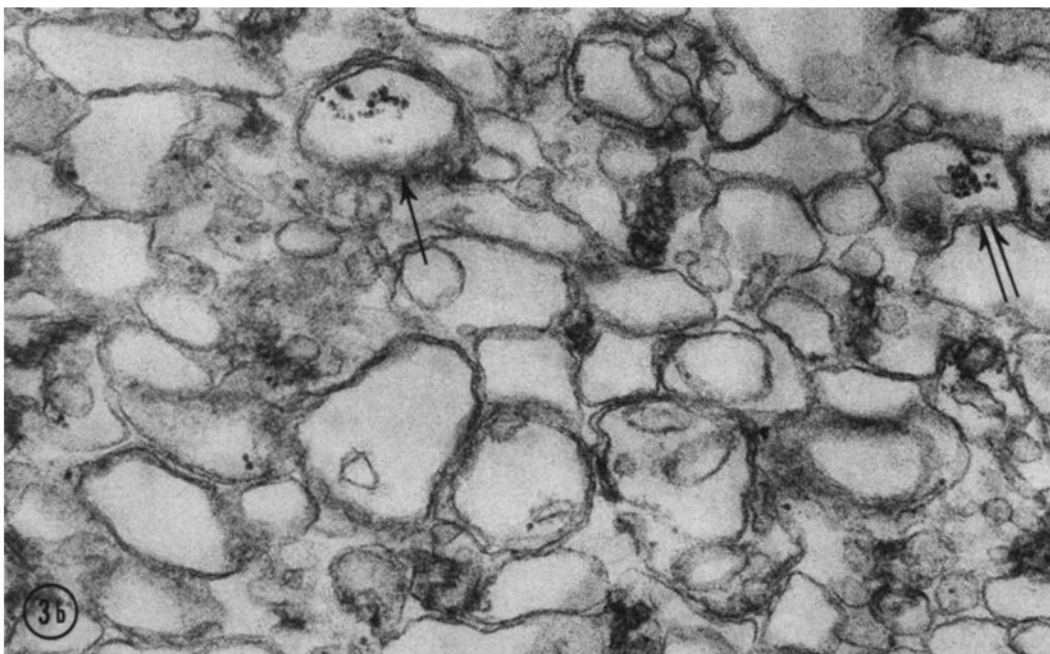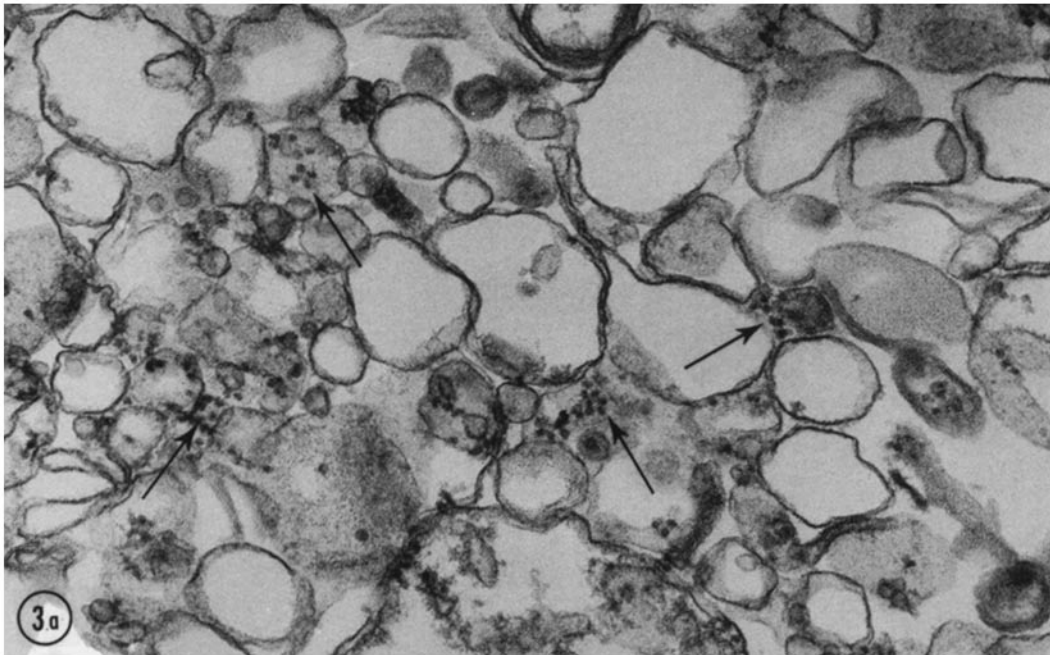
FIGURE 3   Electron micrographs of thin sections through the sonicated mitochondrial vesicle pellet (P25) obtained from an M-3 culture before and after treatment with high salt puromycin.

FIGURE 3 a   P25 before puromycin treatment. This field taken from an area near the top of the fraction shows numerous single-walled vesicles variable in size. When cut tangentially surfaces of the vesicles are seen to be studded with ribosomal particles in clusters (marked by arrows). × 60,000.

FIGURE 3 b   P25 after incubation with puromycin. The field is taken from the upper part of the fraction. Vesicle surfaces are depleted of ribosomal particles. Where present, ribosomes appear within a double-walled vesicle (marked by an arrow) or a complex structure resembling a mitochondrial fragment (marked by a double arrow). × 60,000.

a small proportion ($\frac{1}{6}$ or less) of the total mitochondrial ribosomal subunits. Several lines of evidence suggest that the ribosomal subunits present in S25 represent a pool which is not active in protein synthesis. Ribonucleoprotein particles sedimenting as polysomes are not found in the S25 fraction when the extract is examined directly without incubation in puromycin. Pretreatment of cultures with chloramphenicol to block spontaneous peptide chain termination decreases the ribonucleoprotein content of the S25 fraction. In *poky* where the content of mitochondrial small ribosomal subunits is limiting, the S25 fraction contains only a minor fraction of the small subunit population; this fraction is further diminished when *poky* cultures are treated with chloramphenicol.

In contrast, ribosomes actively engaged in protein synthesis, appear to be associated with vesicles derived from mitochondrial inner membranes. The association depends in part on the presence of a nascent peptide chain. The active ribosomes can be released as subunits by simultaneous treatment with puromycin and high salt buffers. Either treatment alone does not completely release subunits, and the effect of simultaneous treatment is more than additive. In *poky* mitochondria the ribosomal subunits released from P25 by puromycin and high salt show the subunit mass ratio of 2:1 expected for polysomes. This ratio is not encountered in any other fraction nor in a variety of "polysomal" preparations from detergent-lysed mitochondria. In both control and *poky* mitochondria, chloramphenicol pretreatment of cultures increases the content in P25 of puromycin-high salt releasable ribosomal subunits.

There is however a sizable fraction of mitochondrial ribonucleoprotein particles which is retained in the membrane vesicle fraction after puromycin-high salt treatment. Electron microscope studies suggest that this fraction could represent a random population of active and inactive ribosomes trapped within partially disrupted mitochondria. This interpretation is supported by the findings in *poky* where ribosomal subunit ratios in the final P25 pellet are equal to those of intact mitochondria. None the less, the existence of this sizable population of ribonucleoparticles in a form which requires detergent lysis for release makes it impossible to determine what is the true fraction of mitochondrial polysomes which may be associated with membranes. It would also be important to investigate directly the role of the membrane-associated ribosomes in protein synthesis, but here too the significant content of ribonucleoprotein particles in the final vesicle pellet introduces serious difficulties. Before additional progress can be made a more effective means of disrupting mitochondria should be developed, or, alternatively, a subfractionation technique to separate P25 into single-walled vesicle fraction and partially disrupted mitochondrial fraction should be introduced.

In the study of the chloroplast of *Chlamydomonas* already cited here, Chua et al. (10) have pointed out that the association of polyribosomes with thylakoid membranes may reflect their function in membrane biogenesis. It is known from in vitro incorporation studies with isolated mitochondria (16–18) and from in vivo studies using selective inhibitors of mitochondrial protein synthesis (19–22) that the majority of the mitochondrial products are highly hydrophobic peptides tightly associated with the inner membrane. The association between polyribosomes and mitochondrial membranes may provide for on site peptide synthesis at the point of insertion into the membrane of this type of protein.

Linnane and his co-workers have postulated an association between ribosomes and mitochondrial membranes to explain differences between in vivo and in vitro resistance to antibiotics affecting mitochondrial ribosomes of yeast (23). They have also studied the temperature dependence of mitochondrial protein synthesis and correlated discontinuities observed in the Arrhenius plots with phase changes in membrane lipids (24).

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