

# CYTOPLASMIC TYPE 80S RIBOSOMES ASSOCIATED WITH YEAST MITOCHONDRIA

## IV. Attachment of Ribosomes to the Outer Membrane of Isolated Mitochondria

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### ABSTRACT

Growing yeast spheroplasts were shown to have, on the average, four times the number of cytoplasmic ribosomes in contact with the outer mitochondrial membrane compared to starved spheroplasts. Ribosomes in contact with mitochondria in the growing spheroplast preparation, like free cytoplasmic ribosomes, exist primarily as polysome structures. In the starved spheroplast preparation, both mitochondria-bound and free cytoplasmic ribosomes exist primarily as monosomes. Mitochondria isolated from growing spheroplasts in a medium containing 1 mM  $Mg^{++}$  have cytoplasmic ribosomes bound directly to the outer membrane. These ribosomes can be quantitatively removed by washing the mitochondria with 2 mM EDTA. Mitochondria from starved spheroplasts are capable of accepting either free cytoplasmic polysomes or cytoplasmic polysomes extracted from mitochondria. However, the extent of polysome binding to mitochondria was shown to be a direct function of the  $Mg^{++}$  concentration; a smaller percentage of the input polysomes bind as the  $Mg^{++}$  concentration is lowered. At 1 mM  $Mg^{++}$ , neither free cytoplasmic nor mitochondria-bound polysomes bind to mitochondria. Nevertheless, when growing spheroplasts are broken and mitochondria isolated in medium containing 1 mM  $Mg^{++}$ , the mitochondria are seen to have cytoplasmic ribosomes firmly attached to the outer membrane. This result, in addition to our earlier data (Kellems, R. E., and R. A. Butow, 1974. *J. Biol. Chem.* **249**:3304–3310), support the view that cytoplasmic ribosomes attached to the outer membrane of purified mitochondria were attached *in vivo*.

In preparations of mitochondria isolated from growing spheroplasts, ribosomes appear to be bound to specific regions of the outer membrane, namely those regions which are in close association or in contact with the inner mitochondrial membrane. This is particularly evident with mitochondria in a condensed configuration. This finding suggests a mechanism whereby cytoplasmically synthesized mitochondrial protein could be transferred by a process of vectorial translation across both membranes of the organelle.

In eucaryotic cells, the association of cytoplasmic ribosomes with intracellular membranes has been recognized for some time. Ultrastructural examination of eucaryotic cells reveals that ribosomes are bound to the endoplasmic reticulum (ER) and to the outer nuclear membrane. From the standpoint of their morphological, biochemical, and functional properties, ribosomes bound to the ER have been studied most extensively (31). In secretory cells, it is well established that ribosomes bound to the ER function in translational segregation; that is, proteins to be secreted from the cell are translated selectively on this class of cytoplasmic ribosomes. By a process of vectorial translation, the specific translation products are transferred into the lumen of the ER and, subsequently, to the Golgi complex where they are packaged into secretory vesicles which pass to and then fuse with the plasma membrane, thereby releasing the vesicular contents to the extracellular space (23, 4, 28, 8, 9, 25). Serum albumin (37, 40, 13, 7, 42), immunoglobulin (34, 5), and collagen (6, 21) are some examples of proteins known to be selectively synthesized on membrane-bound ribosomes, whereas ferritin (27), myosin (20), and arginase (38) appear to be synthesized on free cytoplasmic ribosomes. Membrane-bound ribosomes also exist in cells with no obvious secretory activity; their function in these cases, however, has not yet been established.

We have recently presented electron micrographs of yeast spheroplasts which show the association of cytoplasmic ribosomes with the outer mitochondrial membrane *in situ* (14). Biochemical studies that we have carried out with isolated preparations of yeast mitochondria and their associated cytoplasmic ribosomes have revealed that these ribosomes have many properties in common with the ribosomes bound to the ER in secretory cells (14-16), and suggest a similarity of function. These results prompted us to undertake an examination of the ultrastructural features of ribosome attachment to the outer mitochondrial membrane.

In this report, we show that cytoplasmic ribosomes are bound *in situ* to the outer mitochondrial membrane in growing yeast spheroplasts and, to a lesser extent, in starved spheroplasts. We present additional evidence to show that in growing spheroplasts ribosomes are bound directly to the outer membrane of isolated mitochondria and, moreover, that the binding is restricted to those regions of the outer membrane which appear to be in

intimate contact with the inner mitochondrial membrane.

## EXPERIMENTAL PROCEDURES

### *Growth of Cells and Preparation of Spheroplasts*

A strain of *Saccharomyces cerevisiae* designated SB-1 was used for this study. Cells were grown to mid-log phase on YPSGal medium (1% yeast extract, 1% Bactopeptone, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.12%  $(\text{NH}_4)_2\text{SO}_4$ , 1% succinic acid, 0.6% NaOH, and 2% galactose) as described earlier (15), harvested by centrifugation, and washed once with distilled water. Cells were converted to spheroplasts by incubation in 1.0 M sorbitol for 1 h at 30°C with a 1% solution of Glusulase (Endo Laboratories, Inc., Garden City, N. Y.). The resulting spheroplasts will be referred to as *nongrowing spheroplasts* since they were exposed to essentially nongrowth conditions for 1 h at 30°C and contain no appreciable quantity of cytoplasmic polysomes (see Results). *Growing* spheroplasts were prepared by resuspending the nongrowing spheroplasts in the original volume of YPSGal containing 0.5 M  $\text{MgSO}_4$  for osmotic stabilization, followed by incubation for 2.5 h at 30°C with shaking. After this procedure, spheroplasts are capable of carrying out macromolecule synthesis (12), and nearly all of their cytoplasmic ribosomes exist as polysomes (see Results).

### *Preparation of Mitochondria*

Spheroplasts, either growing or nongrowing, were harvested by centrifugation at 4°C, and washed twice with ice-cold 1.0 M sorbitol. The following manipulations were carried out at 0°-5°C. The washed spheroplasts were resuspended in 5 ml of 0.5 M sorbitol, 0.010 M Tris-Cl, pH 7.4, 0.001 M  $\text{MgCl}_2$ , 0.005 M  $\text{NH}_4\text{Cl}$ , and 0.001 M mercaptoethanol (designated buffer E) per g of spheroplasts, wet weight, and disrupted by 10 s of homogenization in the Sorvall Omnimixer operating at top speed (Ivan Sorvall, Inc., Newtown, Conn.). Unbroken spheroplasts and cell debris were removed from the homogenate by centrifugation for 10 min at 2,000  $g_{\text{max}}$ . A crude mitochondrial pellet was obtained from the resulting supernatant fraction by centrifugation for 20 min at 12,000  $g_{\text{max}}$ . The crude mitochondrial fraction was washed two times by homogenization in 15 ml 0.8 M sorbitol, 0.010 M Tris-Cl, pH 7.4, 0.001 M  $\text{MgCl}_2$ , 0.005 M  $\text{NH}_4\text{Cl}$ , and 0.001 M mercaptoethanol (designated buffer F), followed by centrifugation for 10 min at 27,000  $g_{\text{max}}$ . In some cases (see Results), mitochondria were washed with 0.8 M sorbitol, 0.010 M Tris-Cl, pH 7.4, and 0.002 M EDTA and are referred to as EDTA-washed mitochondria. In either case, the washed mitochondria were resuspended in buffer F and purified by centrifugation for 90 min at 57,000  $g_{\text{max}}$  through a 20-60% sucrose gradient prepared in 0.010 M Tris-Cl, pH 7.4, 0.001 M  $\text{MgCl}_2$ , 0.005 M  $\text{NH}_4\text{Cl}$ , and 0.001 M

mercaptoethanol. The brown mitochondrial band was carefully removed with a syringe, diluted with buffer F, and pelleted.

In all cases above, the buffers contained 100  $\mu\text{g}/\text{ml}$  cycloheximide.

### *Preparation of Samples for Electron Microscopy*

Samples were chemically fixed by incubation for 2 h at  $0^\circ\text{C}$  with a 3% solution of glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate. After initial fixation, the samples were washed several times over a period of at least 2 h with 0.1 M phosphate buffer, pH 7.2, containing 10% sucrose and then postfixed with 2% osmium tetroxide, also buffered at pH 7.2 with 0.1 M phosphate. In each case, the phosphate buffers contained 50  $\mu\text{g}/\text{ml}$  of  $\text{CaCl}_2$ . Samples were subsequently dehydrated and embedded in Maraglas epoxy resin (The Marblette Co., Div. of Allied Products Corp., Long Island City, N. Y.). Sections 300–400  $\text{\AA}$  thick were cut on an LKB model 8801 ultratome equipped with a diamond knife (LKB Instruments, Inc., Rockville, Md.). Sections were collected onto copper grids, stained consecutively with uranyl acetate and lead citrate (29), and examined on either a Hitachi HU11B-2 or Philips EM-300 electron microscope.

### *Polysome Binding to Isolated Mitochondria*

Mitochondria-bound and free cytoplasmic polysomes used in the *in vitro* binding experiments (see Results) were prepared as previously described (14), except that in this case the concentration of  $\text{MgCl}_2$  and  $\text{NH}_4\text{Cl}$  was reduced 10-fold. *In vitro* polysome binding to isolated mitochondria was determined by the rapid assay procedure described before (16).

### *Determination of Mitochondrial Density*

In cases where mitochondrial density was determined, the procedure described before was used (16), except that the sucrose gradient used here was 20–60% sucrose containing 0.010 M Tris-Cl, pH 7.4, 0.001 M  $\text{MgCl}_2$ , 0.005 M  $\text{NH}_4\text{Cl}$ , 0.001 M mercaptoethanol, and 100  $\mu\text{g}/\text{ml}$  cycloheximide.

### *Analytical Procedures*

Cytochrome oxidase was assayed by the procedure described by Wharton and Tzagoloff (41). Protein concentrations were determined by the method of Lowry et al. (19). The radioactivity of aqueous samples was determined using the toluene-Triton X-100 scintillant of Patterson and Greene (24). The growth of cells was measured as optical density using a Klett photometer with a no. 66 filter (Klett Manufacturing Co., Inc., New York).

## RESULTS

### *Electron Micrographs of Growing and Starved Spheroplasts*

As part of our efforts to understand the functional significance of cytoplasmic ribosomes bound to mitochondria in yeast, we previously determined what effect various changes in the metabolic state of the cell had on the amount of cytoplasmic ribosomes associated with mitochondria (16). Briefly, we found that there was a significant reduction in the amount of bound cytoplasmic ribosomes recovered with purified mitochondria isolated from starved cells or cells in stationary phase compared with mitochondria isolated from growing cells. Furthermore, it was possible to show that a change in the quantity of mitochondria-bound cytoplasmic ribosomes could be correlated directly with a change in the isopycnic density of the isolated mitochondria (16). In an effort to determine if such changes in the quantity of cytoplasmic ribosomes bound to mitochondria occurred *in situ*, we examined thin sections of growing and starved yeast spheroplasts by electron microscopy. The results presented in Figs. 1 and 2 show clearly the difference between growing and starved preparations regarding the extent to which cytoplasmic ribosomes are associated with the outer mitochondrial membrane. Whereas only an occasional ribosome appears in contact with the other membrane in the starved preparation (Fig. 2), ribosomes are seen to occur in obvious stretches or runs along the outer mitochondrial membrane in the growing preparation and, in some cases, cover nearly the entire mitochondrial periphery (Fig. 1).

In order to document these observations more quantitatively, about 50 mitochondrial profiles, randomly chosen from both growing and starved spheroplasts, were compared with regard to the number of cytoplasmic ribosomes in apparent contact with the outer mitochondrial membrane. In each case the number of ribosomes that we judged to be touching the membranes was determined as a function of the contour length of the mitochondrion. Those regions of the outer mitochondrial membrane not clearly delineated in the section were not used in making these calculations. The results of the determinations presented in Fig. 3 show that mitochondria from growing spheroplasts have, on the average, four times more ribosomes in contact per unit contour length of outer mitochondrial membrane than do mitochondria

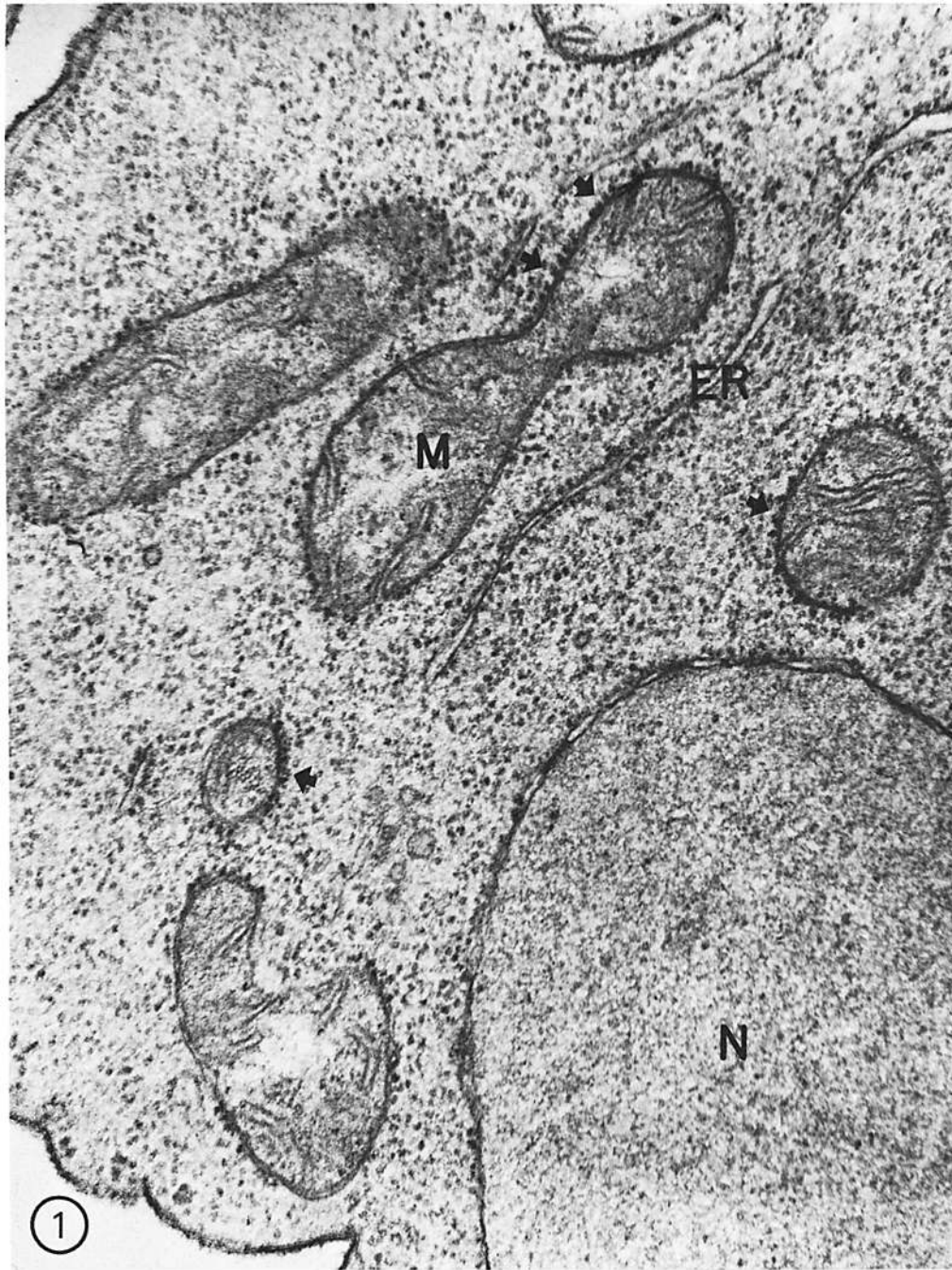


FIGURE 1 An electron micrograph showing a representative thin section through a growing yeast spheroplast. Numerous mitochondria are readily apparent in the cytoplasm of the cell. Note particularly the apparent alignment of cytoplasmic ribosomal particles on regions of the outer mitochondrial membrane (arrows). *M*, mitochondria; *N*, nucleus; *ER*, endoplasmic reticulum.  $\times 65,000$ .



FIGURE 2 An electron micrograph showing a representative thin section through a starved yeast spheroplast. Note that cytoplasmic ribosomal particles do not appear aligned along the outer mitochondrial membrane as in Fig. 1. *M*, mitochondria.  $\times 40,000$ .

dria in starved spheroplasts (Fig. 3). Thus, these results not only confirm our previous results obtained with isolated mitochondria showing that the quantity of cytoplasmic ribosomes associated with the mitochondria changes in response to changes in the metabolic state of the cell (16), but, in addition, demonstrate that a reversible association of cytoplasmic ribosomes with the outer

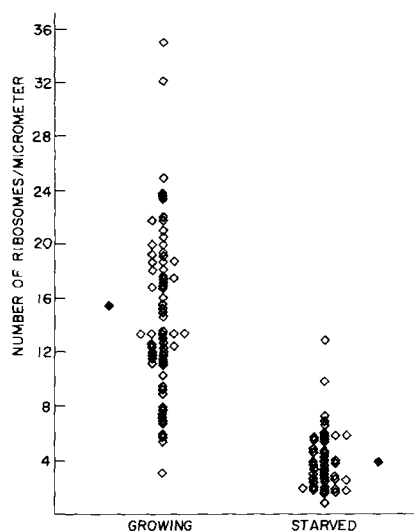


FIGURE 3 A comparison of the number of cytoplasmic ribosomal particles associated with mitochondria of growing and starved spheroplasts. About 50 mitochondrial profiles each were randomly chosen from electron micrographs of thin sections of growing and starved spheroplasts. The number of cytoplasmic ribosomal particles in contact with the outer mitochondrial membrane was determined in each case as a function of mitochondrial contour length. Regions where the outer mitochondrial membrane was not clearly delineated were not considered in these determinations. Each open diamond represents one mitochondrion. The average value for the growing and starved preparations is indicated by the filled diamonds.

mitochondrial membrane occurs *in vivo*. We consider this latter result highly significant since it seems unlikely that such differences between growing and starved preparations would obtain if ribosome contact with the outer mitochondrial membrane were the result *only* of random interactions. Taken together, these data suggest that cytoplasmic ribosome binding to the outer mitochondrial membrane is a true morphological feature of growing yeast spheroplasts.

The presence of runs or stretches of ribosomes along the outer mitochondria membrane of growing spheroplasts suggests that these ribosomes are present as polysome structures. In order to determine if this is indeed the case and to verify that our growing and starved spheroplasts are characterized by active and depressed rates of protein synthesis, respectively, we prepared cytoplasmic ribosomes from isolated mitochondria and the postmitochondrial supernatant fractions of growing and starved spheroplasts. As shown in Fig. 4 A

and C, both mitochondria-bound and free cytoplasmic ribosomes exist almost entirely as polysomes when prepared from isolated mitochondria or the postmitochondrial supernatant fractions of growing spheroplasts. On the other hand, virtually all of the cytoplasmic ribosomes prepared from these fractions of starved spheroplasts are present as monosomes (Fig. 4 B and D). These results are in accord with the interpretation that cytoplasmic ribosomes detach from the outer mitochondrial membrane as a result of ribosome runoff from messenger RNA (mRNA) and also strengthen the possibility that the runs of ribosomes seen attached to mitochondria in growing spheroplasts are polysomal structures.

In this regard it is of interest to note that cytoplasmic ribosomes bound to mitochondria in yeast behave like membrane-bound ribosomes in higher eucaryotic cells: in both cases, conditions

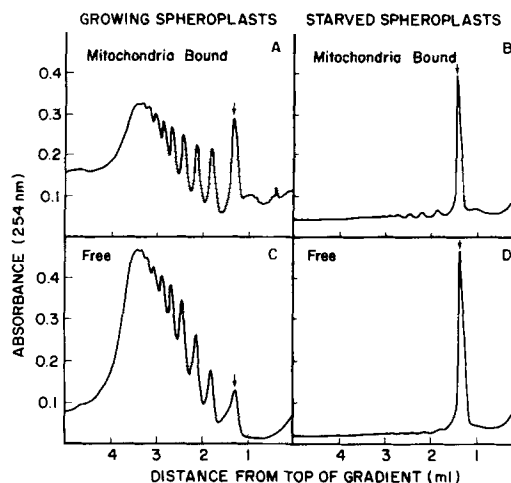


FIGURE 4 Optical density profiles of mitochondria-bound and free cytoplasmic polysomes prepared from growing and starved yeast spheroplasts. Polysomes were prepared as previously described (20) from isolated mitochondria (A and B) and the postmitochondrial supernatant fraction (C and D) of growing and starved spheroplasts. Optical density profiles were determined after centrifugation for 40 min at  $227,861 g_{max}$  in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) through a 10–50% sucrose gradient containing 0.010 M Tris-Cl, pH 7.4, 0.010 M  $MgCl_2$ , 0.050 M  $NH_4Cl$ , and 0.001 M mercaptoethanol. Gradients were scanned for absorbance at 254 nm using an ISCO model 183 density gradient fractionator equipped with a model 224 UV flow analyzer (Instrumentation Specialties Co., Lincoln, Neb.). The position of the 80S monosome is indicated by an arrow.

which permit a decrease in protein synthesis due to decreased initiation or ribosome runoff result in a decrease in the amount of membrane-bound ribosomes (18, 10, 17).

### *Ribosomes are Bound to the Outer Membrane of Isolated Mitochondria*

Fig. 5 shows a representative field of mitochondria isolated in the presence of 1 mM  $Mg^{++}$  from growing spheroplasts. This result shows unambiguously that ribosomes are attached directly to the outer mitochondrial membrane. Although some membrane fragments can be seen which are not identifiable as mitochondria or as structures derived from mitochondria, the result rules out the possibility that membrane-bound cytoplasmic ribosomes which are recovered with purified mitochondria are due entirely to contamination of the preparation by rough ER. Moreover, the fact that the mitochondria have been washed several times and centrifuged to isopycnic equilibrium before fixation and preparation for ultrastructural analysis, suggests that the cytoplasmic ribosomes are quite securely anchored to the outer membrane. In this connection, we have previously provided biochemical evidence that two types of interactions serve to anchor cytoplasmic ribosomes to mitochondria: one is an ionic interaction between the ribosome and the membrane which is sensitive to KCl, and the other is a puromycin-sensitive interaction presumably involving the nascent polypeptide chain (14).

Another procedure for removing ribosomes from membranes which allows the membranes to remain relatively intact involves the use of chelating agents (33, 26, 30). We have found that when mitochondria are prepared from either growing or starved spheroplasts in the presence of buffers containing 2 mM EDTA, the recovery of bound cytoplasmic ribosomes is drastically reduced. As a result, the isopycnic density of the mitochondria decreases and the mitochondria have a much greater capacity to bind exogenously added ribosomes *in vitro* than do mitochondria isolated in the presence of  $Mg^{++}$  from growing cells (16). The effect of EDTA in removing bound ribosomes from the outer mitochondrial membrane is shown by the electron micrograph presented in Fig. 6. It is evident that the removal of attached ribosomes has been almost quantitatively effected.

### *Effect of $Mg^{++}$ Concentration on Polysome Binding to Isolated Mitochondria*

Mitochondria stripped of bound cytoplasmic ribosomes by washing with 2 mM EDTA will bind added ribosomes or polysomes *in vitro* when incubated in a medium of low ionic strength. In an effort to understand the nature of the ribosome-membrane interaction, we have previously studied the effect of various conditions on ribosome or polysome binding to mitochondria *in vitro* (16). We have found, for example, that the attachment of polysomes to mitochondria does not occur in the presence of either 350 mM KCl or  $10^{-4}$  M aurintricarboxylic acid although cells opened and fractionated under these conditions show, at most, only a slight reduction in the recovery of cytoplasmic ribosomes with the mitochondria. We have taken these results as evidence that cytoplasmic ribosomes were attached to mitochondria *in vivo* before exposure to these reagents.

As a continuation of these investigations, we have studied the effect of the  $Mg^{++}$  concentration on the binding of polysomes to isolated mitochondria. In these experiments, we used mitochondria isolated from starved spheroplasts since the number of ribosomes attached to such mitochondrial preparations is greatly reduced compared with mitochondria prepared from growing cells, thereby exposing nascent ribosome binding sites without the use of chelating agents. Cytoplasmic polysomes were incubated with isolated mitochondria in the presence of various concentrations of  $Mg^{++}$ , and the percentage of input polysomes which became attached was determined. As shown in Fig. 7, polysome binding to mitochondria is quite sensitive to the  $Mg^{++}$  concentration, becoming drastically reduced as the concentration is lowered. At 1 mM  $Mg^{++}$ , the binding to mitochondria of cytoplasmic polysomes prepared either from isolated mitochondria or from the postmitochondrial supernatant fraction is completely eliminated *in vitro*. Nevertheless, mitochondria isolated in the presence of 1 mM  $Mg^{++}$  from growing spheroplasts retain bound ribosomes. These results provide additional evidence that the membrane-bound ribosomes recovered with isolated mitochondria are, in fact, attached *in situ*. These data suggest that by isolating mitochondria under conditions of low  $Mg^{++}$ , any nonphysiological association of polysomes with the outer mitochondrial membrane would be minimized; consequently, with the exception of the EDTA-washed mitochondria, all

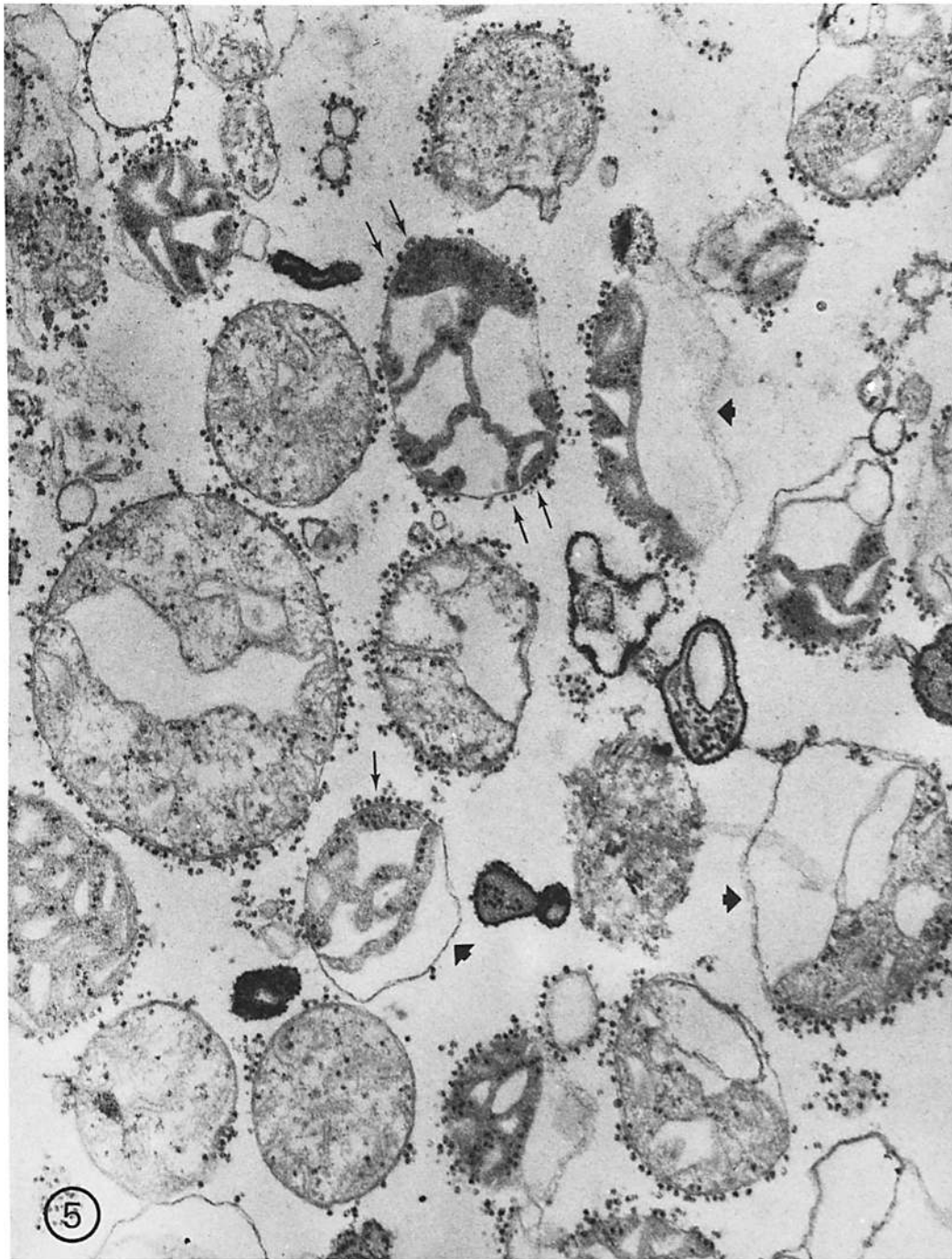
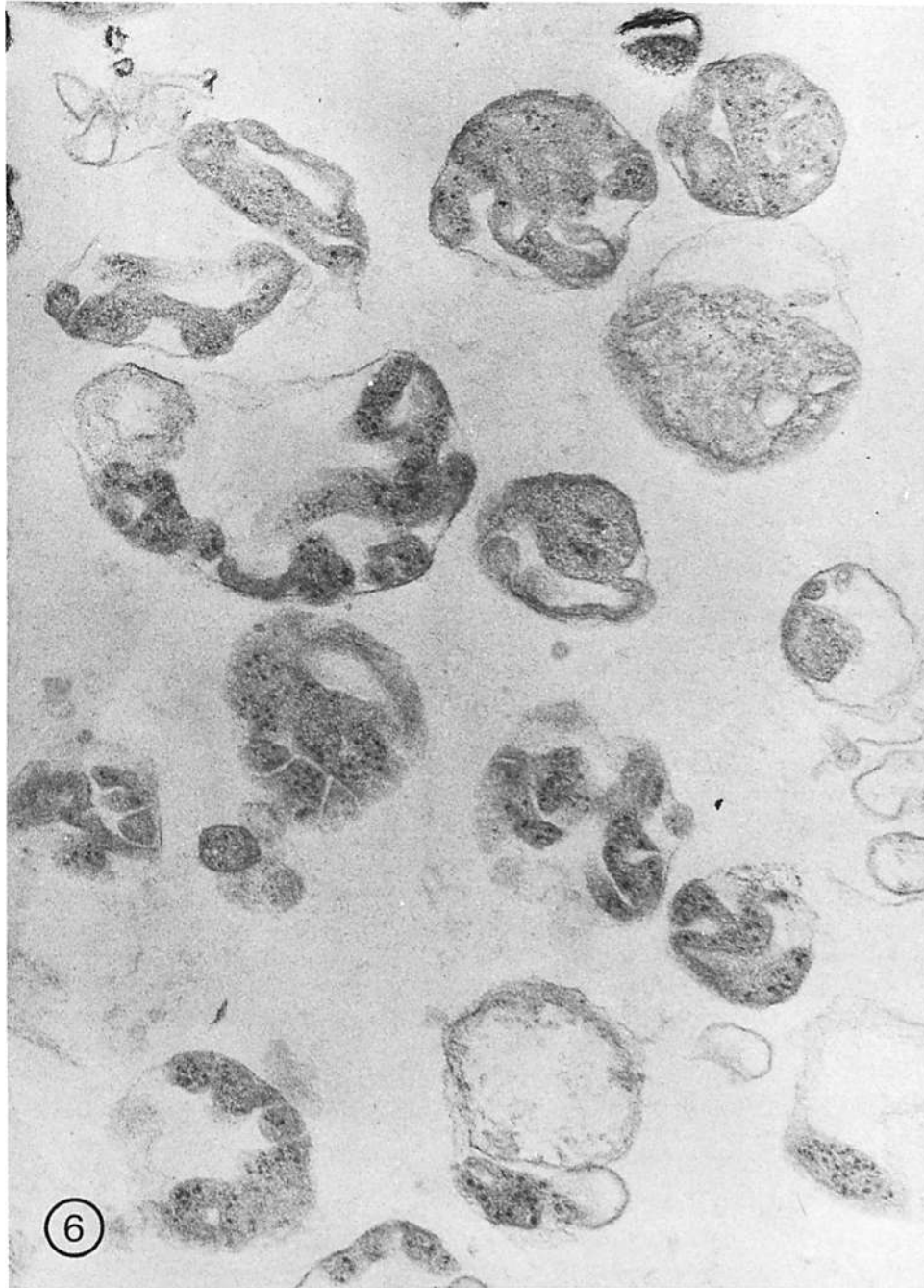


FIGURE 5 Representative field of mitochondria isolated from growing spheroplasts. Cells from a log phase culture (Klett 100) were converted to growing spheroplasts as described under Experimental Procedures. After 2.5 h the growing spheroplasts received 200  $\mu\text{g/ml}$  of cycloheximide and 10 min later were harvested. Mitochondria were isolated from the growing spheroplasts, purified by centrifugation to isopycnic equilibrium in a 20-60% sucrose gradient, and prepared for electron microscope examination as outlined under Experimental Procedures. Note the attachment of cytoplasmic ribosomes to regions of the outer mitochondrial membrane (arrows). Regions of the outer mitochondrial membrane not in apparent contact with the inner membrane are devoid of attached ribosomes (arrowheads).  $\times 61,750$ .





**FIGURE 6** An electron micrograph of EDTA-washed mitochondria. Sucrose gradient purified mitochondria were isolated in the presence of 0.8 M sorbitol, 0.010 M Tris-Cl, pH 7.4, and 0.002 M EDTA from growing spheroplasts and prepared for electron microscope examination as described under Experimental Procedures. Mitochondria prepared in this manner do not have attached cytoplasmic ribosomes.  $\times 67,200$ .

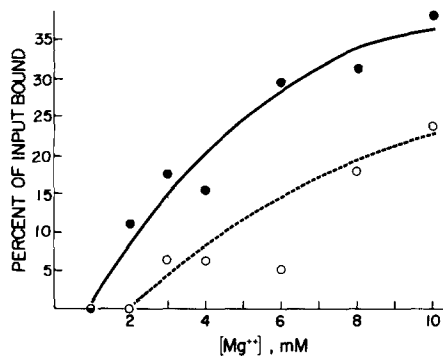


FIGURE 7 Polysome binding to isolated mitochondria as a function of the  $Mg^{++}$  concentration. Mitochondria were prepared from starved spheroplasts and incubated with either 0.75- $A_{260}$  units of cytoplasmic polysomes extracted from isolated mitochondria (●—●) or 0.5- $A_{260}$  units of free cytoplasmic polysomes isolated from the postmitochondrial supernatant fraction (○—○). The incubations were performed at 30°C for 15 min in buffer F containing increasing concentrations of  $MgCl_2$ . Polysome binding to 0.5 mg mitochondrial protein was determined by the rapid assay procedure described earlier (21).

other mitochondrial preparations described in this report were isolated in the presence of 1 mM  $Mg^{++}$ . Whether there is any significance to the small difference in efficiency of binding to mitochondria between free and bound cytoplasmic polysomes at the higher  $Mg^{++}$  concentrations is not clear at this time.

Examination of the electron micrographs of isolated mitochondria shown in Fig. 5 and at higher magnification in Fig. 8 reveals an unexpected and provocative feature of ribosome binding to the outer mitochondrial membrane. It is evident from inspection of these micrographs that ribosomes appear to be attached preferentially to those regions of the outer membrane where the inner and outer membranes are in intimate contact. Those regions of contact or possibly fusion between the inner and outer membranes are most readily visualized with mitochondria in a *condensed* or de-energized state (Fig. 8 *a* and *c*) as compared to the *orthodox* or energized configuration (Fig. 8 *b* and *d*). These configurations correspond to those described by Hackenbrock for rat liver mitochondria (11). We note that in those instances where a mitochondrion is in a condensed configuration and, in particular, where the inner membrane has condensed to one side of the mitochondrion, the region of the outer membrane away from the condensed matrix and inner mem-

brane is essentially devoid of bound ribosomes (Fig. 8 *a*). Moreover, even in those areas where the bulk of the inner membrane is in close proximity to the outer membrane, ribosomes appear clustered primarily at localized regions of contact (Fig. 8 *c*). This situation is to be contrasted with mitochondria in a more orthodox configuration where proximity between the inner and outer membranes occurs continuously over a relatively large distance. In this case, ribosomes are attached all along that region of the outer membrane (Fig. 8 *b* and *d*).

With these observations in mind, we are led to conclude that in the case of yeast mitochondria, cytoplasmic ribosomes are attached specifically to contact points. Furthermore, this finding not only demonstrates that ribosome attachment is specific for certain regions of the outer membrane but, in addition, immediately suggests a possible mechanism for transferring completed polypeptides directly through two membrane systems via a process of vectorial translation.

## DISCUSSION

The results presented here demonstrate that in yeast a reversible association occurs *in vivo* between cytoplasmic ribosomes and the outer mitochondrial membrane. This conclusion is readily apparent from a comparison of thin sections of growing and starved spheroplasts. There is, on the average, four times the number of ribosomes in contact with the outer mitochondrial membrane in the growing compared with starved spheroplast preparation. These results are in accord with our previous observations (16) that when cellular protein synthesis is depressed, for example, by starvation of a growing culture of cells or by the use of a temperature-sensitive mutant defective in the initiation of translation at the nonpermissive temperature, the quantity of cytoplasmic ribosomes associated with purified yeast mitochondria decreases markedly.

From the nature of the polysome profiles shown in Fig. 4 we conclude that ribosomes which are bound to the outer mitochondrial membrane in actively growing yeast spheroplasts are bound predominately as polysomes. Moreover, the data suggest that ribosomes detach from the outer mitochondrial membrane after a round of translation and reattach upon initiation of a new round of protein synthesis. Our general observations are reminiscent of the behavior of membrane-bound ribosomes in a variety of other systems where the

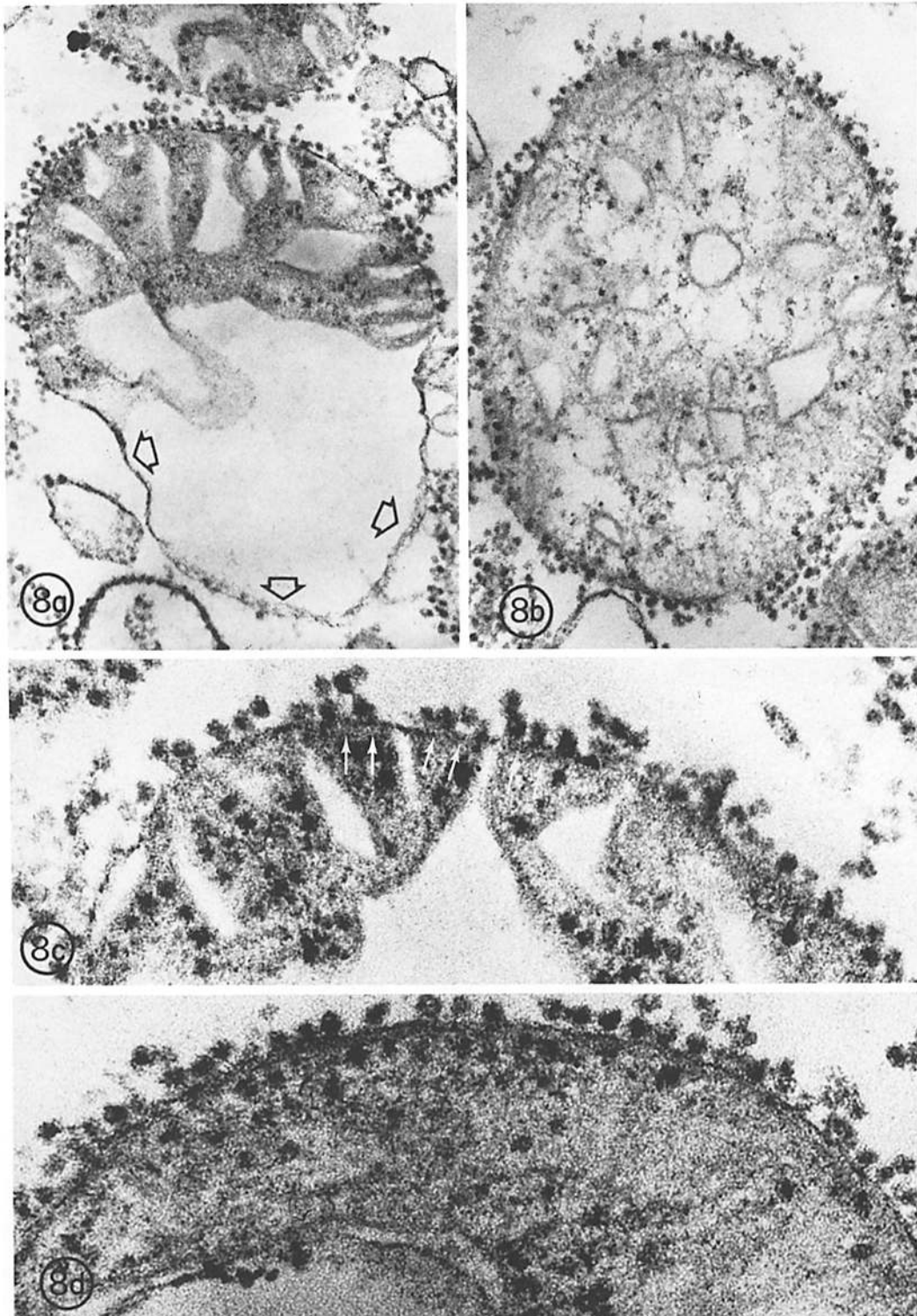


FIGURE 8 Ultrastructural features of cytoplasmic ribosome attachment to the outer mitochondrial membrane. Mitochondria are represented in condensed (*a* and *c*) and orthodox (*b* and *d*) configurations. White arrows indicate clustering of ribosomes on regions of the outer membrane which are in apparent contact with the inner membrane. Few ribosomes are attached to the outer membrane in noncontacted regions (open arrows). *a*,  $\times 64,000$ ; *b*,  $\times 70,200$ ; *c*,  $\times 154,000$ ; *d*,  $\times 160,000$ .

extent of association of ribosomes with membranes appears related to the extent of engagement of ribosomes with mRNA. The details of the mechanism of assembly of membrane-bound ribosomes, however, are not yet completely understood. While it seems clear that in these systems ribosome binding to membrane occurs through the large subunit (3) and that nascent polypeptide plays a role in securing ribosomes to membranes (1), it is not known at what stage in the translation sequence or what species of the translation complex (e.g. mRNA, nascent polypeptide chain, or ribosome) confers ribosome specificity for the membrane.

In evaluating the significance of the distribution of cytoplasmic ribosomes between free and membrane-bound states, it is useful to consider conditions which effect an apparent reversible association between ribosomes and membranes and, if possible, to correlate the presence of bound ribosomes with a particular functional state of the cell. For example, the development of an extensive rough ER system in cells actively engaged in the synthesis and secretion of extracellular protein (22, 2) is in accord with the widely accepted mechanism of the secretory process, i.e., that of vectorial translation of specific polypeptides into the lumen of the ER for later transport from the cell. It is more difficult, however, to relate structure to function in the case of membrane-bound ribosomes in cells with no obvious secretory activity. It has been suggested that the topographical segregation of cellular protein synthesis (that is, between free and membrane-bound ribosomes) may be a means of achieving intracellular segregation and compartmentalization of specific proteins (39). The remarkable similarities between the properties of ribosomes attached to the ER of secretory cells and of ribosomes bound to the outer mitochondrial membrane in yeast, including their capability to carry out vectorial translation (10), suggest a similarity of function; in the latter, that function would be to transfer cytoplasmically synthesized mitochondrial proteins into the organelle.

Studies on the binding of the ribosomes to ER membranes first stripped of endogenously bound ribosomes show that the membranes have a limited number of ribosome binding sites. These putative specific sites are labile (35) and can be readily destroyed by limited proteolysis (36). Recently, it has been estimated that the association constant between the 60S subunit and the specific ribosome binding sites on the rough ER membranes is  $2 \times$

$10^{-8}$  M (32). Thus, it appears that the rough ER membranes possess specific ribosome accepting sites, but little data are available on the organization of these sites on the ER membrane or their particular molecular features.

Our observations (Figs. 5 and 8) showing morphological specificity of ribosome binding to the outer mitochondrial membrane offer new possibilities to study details of ribosome-membrane interactions. Importantly, the data we present in Figs. 5 and 8 show that the distribution of ribosomes on the outer mitochondrial membrane is not random. Few ribosomes are seen bound to regions of the outer mitochondrial membrane that are not in intimate association or contact with the inner membrane. This is particularly evident when mitochondria are in a condensed configuration. There are two main possibilities which might be considered to account for this observation. First, the apparent specificity of ribosome binding may reflect specific sites on the outer mitochondrial membrane. Furthermore, these putative specific sites would not be randomly distributed but located preferentially in regions of the outer membrane which are close to or in contact with the inner mitochondrial membrane. However, in view of the fact that it is possible to observe mitochondrial profiles in an orthodox configuration which are studded with ribosomes all around the mitochondrial periphery and, since it is possible to convert orthodox mitochondria to a condensed configuration and vice versa (11), this simple picture does not seem likely. A second possibility is that the sites to which ribosomes bind to the outer membrane are not specific insofar as they represent unique ribosome binding sites; rather, the apparent specificity would be the consequence of the attachment and disposition of the nascent polypeptide chain relative to the inner and outer membranes. The requirement for the nascent polypeptide chain in the attachment of ribosomes to the outer mitochondrial membrane is remarkably similar to that of membrane-bound ribosomes in liver; in both cases, only 30–40% of the ribosomes can be detached by high concentrations of KCl, and quantitative removal of bound ribosomes requires the combined action of puromycin and high concentrations of KCl (1). In the case of ribosomes bound to the ER, these observations reflect their function: the transfer of growing polypeptides across the membrane. Since cytoplasmic ribosomes bound to yeast mitochondria also have the capacity to carry out vectorial translation

(14) and display a similar KCl- and puromycin-dependent release from the membrane, the apparent specificity of binding of ribosomes may be the consequence of the translocation of nascent polypeptides across the outer membrane to the inner membrane which are in contact or possibly fused together. Although presently we cannot decide between these models, the implication that ribosomes bound to the outer mitochondrial membrane in yeast might function to transport mitochondrial proteins into the organelle is clear.

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