

MOBILITY OF RIBOSOMES BOUND TO MICROSOMAL MEMBRANES

A Freeze-Etch and Thin-Section Electron Microscope Study of the Structure and Fluidity of the Rough Endoplasmic Reticulum

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ABSTRACT

The lateral mobility of ribosomes bound to rough endoplasmic reticulum (RER) membranes was demonstrated under experimental conditions. High-salt-washed rough microsomes were treated with pancreatic ribonuclease (RNase) to cleave the mRNA of bound polyribosomes and allow the movement of individual bound ribosomes. Freeze-etch and thin-section electron microscopy demonstrated that, when rough microsomes were treated with RNase at 4°C and then maintained at this temperature until fixation, the bound ribosomes retained their homogeneous distribution on the microsomal surface. However, when RNase-treated rough microsomes were brought to 24°C, a temperature above the thermotropic phase transition of the microsomal phospholipids, bound ribosomes were no longer distributed homogeneously but, instead, formed large, tightly packed aggregates on the microsomal surface. Bound polyribosomes could also be aggregated by treating rough microsomes with antibodies raised against large ribosomal subunit proteins. In these experiments, extensive cross-linking of ribosomes from adjacent microsomes also occurred, and large ribosome-free membrane areas were produced. Sedimentation analysis in sucrose density gradients demonstrated that the RNase treatment did not release bound ribosomes from the membranes; however, the aggregated ribosomes remain capable of peptide bond synthesis and were released by puromycin. It is proposed that the formation of ribosomal aggregates on the microsomal surface results from the lateral displacement of ribosomes along with their attached binding sites, nascent polypeptide chains, and other associated membrane proteins. The inhibition of ribosome mobility after maintaining rough microsomes at 4°C after RNase, or antibody, treatment suggests that the ribosome binding sites are integral membrane proteins and that their mobility is controlled by the fluidity of the RER membrane. Examination of the hydrophobic interior of microsomal membranes by the freeze-fracture technique revealed the presence of homogeneously distributed 105-Å intramembrane particles in control rough microsomes. However, aggregation of ribosomes by RNase,

or their removal by treatment with puromycin, led to a redistribution of the particles into large aggregates on the cytoplasmic fracture face, leaving large particle-free regions.

The endoplasmic reticulum (ER) is an intracellular membrane system richly endowed with important enzymatic activities that play a major role in the biosynthesis, modification, and subcellular distribution of specific classes of proteins. Morphologically distinct regions in the ER membranes (rough endoplasmic reticulum (RER); cf. reference 47) bear ribosomes bound to specific sites on their cytoplasmic surface (11, 62, 70). These ribosomes are engaged in the synthesis of secretory proteins, proteins of both rough and smooth ER membranes, and proteins which are retained within other subcellular organelles (cf. references 47 and 64).

Although the detailed architecture of the binding sites for ribosomes in the RER membranes is unknown, integral membrane proteins are presumed to be important constituents of these sites (11, 29, 30). Large subunits of bound ribosomes interact with the binding sites (66) via salt-sensitive linkages (2), and, in addition, nascent polypeptides which emerge from the large subunits play an important role in firmly anchoring active bound ribosomes to the membranes (2, 63, 66).

In rat liver rough microsomes (RM), a major fraction of nascent chains in bound ribosomes corresponds to secretory proteins (48, 57). These are vectorially discharged into the ER lumen by a mechanism which requires the maintenance, throughout most of the translation cycle, of an intimate and firm association between the ribosome and their nascent chains on one side and the microsomal membrane on the other (2, 58, 59, 63).

It has been established that, in biological membranes, both lipids (28, 68), and membrane proteins (15, 20, 22, 27, 49, 55, 75) have the capacity for extensive lateral movement in the plane of the membrane. These properties of membrane components have been reviewed and incorporated into a general model of membrane organization (19, 71, 72).

The experiments presented in this paper were designed to study the possibility that ribosomes, their binding sites, and associated membrane components are, or can become, mobile in the plane of a fluid RER membrane (18, 21, 56, 60, 61, 74, 78, 81, 82). Not only would this facilitate the

relative displacement of ribosomes and mRNA with respect to each other during translation, but it could also serve to insure the availability of binding sites for ribosomes in the vicinity of newly formed initiation complexes during the assembly of bound polysomes.

Since most membrane-bound ribosomes are part of polysomes, we introduced a mild RNase treatment designed to cleave the mRNA and allow the movement of individual ribosomes. The RNase treatment also damaged the surface of bound ribosomes which rendered them adhesive to one another. In an alternative procedure, RM were incubated with antibodies against 60S ribosomal proteins which allowed the cross-linking of adjacent bound polysomes through their large ribosomal subunits. The distribution of bound ribosomes on RNase- or antibody-treated RM was then studied by thin-section electron microscopy as well as by freeze-etching (38, 73), a powerful technique which splits biological membranes into complementary halves (12). After etching, the simultaneous visualization of both internal features and membrane surfaces is possible (51, 76, 77).

Our observations indicate that membrane-bound ribosomes, their attached binding sites, and associated intramembrane components are capable of exhibiting extensive lateral displacements in the plane of the rough microsomal membrane. Preliminary reports on aspects of this work have been presented (41, 42, 65).

MATERIALS AND METHODS

Cell fractionation

All solutions used were made up in double-distilled de-ionized water and then Millipore (Millipore Corp., Bedford, Mass.) filtered (0.45 μm pore size; 1.2 μm pore size filters were used for concentrated sucrose solutions). The composition of low salt buffer (TKM) was 50 mM triethanolamine-HCl, pH 7.5 at 20°C, 25 mM KCl, and 5 mM MgCl_2 . High salt buffer (HSB) had the same composition as TKM, but the KCl concentration was adjusted to 0.5 M. All centrifugations were carried out in Beckman ultracentrifuges (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), at 4°C, unless stated otherwise.

Male Sprague-Dawley rats (120–150 g) fasted for

approx. 18 h were used to prepare liver microsomes according to the procedure of Adelman et al. (1) with the following modifications (introduced by Dr. J. Kruppa): the postmitochondrial supernate was adjusted to 1.35 M sucrose and layered over a step gradient consisting of 5 ml of 2.0 M sucrose-TKM and 5 ml of 1.55 M sucrose-TKM containing 1.5 ml of rat liver high-speed supernate (7) as an inhibitor of RNase. After centrifugation for 16 h at 40,000 rpm in a Ti60 rotor, the RM were collected from the 1.55–2.0 M interface and diluted 1:1 with HSB (2× concn) before sedimentation (30 min at 30,000 rpm in a Ti60 rotor) to obtain the final RM preparation.

RNase Treatment

Aliquots (1 ml) of a RM suspension in TKM (2–3 mg protein/ml) pipetted into small (1.5 ml) conical polypropylene microcentrifuge tubes received pancreatic RNase (0.5 mg/ml in 0.1 M NaCl, 10 mM Tris-HCl pH 7.5, and 1 mM EDTA) to a final concn of 10 µg/ml. After a 10-min incubation at 4°C, the RM were recovered by centrifugation for 2 min at 4°C in an Eppendorf 3200 microcentrifuge (Brinkman Instruments, Westbury, N.Y.), resuspended in TKM-containing high-speed supernate (4:1), washed once by sedimentation and resuspended in 1 ml of the same solution. Aliquots (1 ml) of the treated microsomes were then incubated at 4°, 24°, or 37°C as indicated in the text. Control samples (not treated with RNase) or RM treated with inactivated RNase (83) were processed in an identical manner.

Ribosomal Removal

To prepare microsomal vesicles stripped of ribosomes, RM were incubated in HSB containing 2.5 mM MgCl₂ and 10⁻³ M puromycin (2) for 30 min at 4°C followed by 30 min at 24°C. After centrifugation (2 min in the Eppendorf centrifuge) the supernate was removed by aspiration and the membranes were fixed in glutaraldehyde-HSB as described below.

Preparation of Antibody against

Large Ribosomal Subunits

To prepare antibodies against 60S ribosomal proteins, bound ribosomes from RM were removed and dissociated into subunits by the puromycin-HSB procedure (9). Small and large ribosomal subunits were then separated by centrifugation at 20°C through 10–25% sucrose density gradients (containing HSB) in an SW41 rotor for 2 h at 40,000 rpm.

Purified large ribosomal (60S) subunits were mixed with Freund's complete adjuvant, and 0.5 mg of ribosomal protein was injected into rabbits at weekly intervals for 3 wk followed by two further injections at bi-weekly intervals. Two weeks after the final injection, the rabbits were bled and an anti-60S gamma globulin fraction (IgG) was prepared by ammonium-sulfate precipitation and *O*-(diethylaminoethyl)cellulose (DEAE-cellu-

lose) column chromatography according to standard procedures (80).

Procedures for Electron Microscopy

Microsome samples were fixed for 15 min at 4°C, 24°C, or 37°C by adding 0.5 ml of 4% glutaraldehyde-TKM maintained at the appropriate temperature to 1 ml of the microsomal suspension. Fixed microsomes were sedimented in the Eppendorf centrifuge and maintained in fixative for an additional 15-min period. The fixed microsomes were then washed overnight at 4°C in TKM. Samples to be embedded for thin sectioning were post-fixed for 1 h in 1% OsO₄, 0.1 M cacodylate and stained in block with 1% uranyl acetate before dehydration and embedding in Epon 812. Thin sections were examined in a Philips 301 electron microscope operating at 80 kV.

For freeze-fracturing, glutaraldehyde-fixed microsomes were also washed overnight in TKM, then resuspended in 10% glycerol-TKM and maintained in this medium for 1 h. To break up any clumps and facilitate handling, the fixed microsomes were gently homogenized in glycerol-TKM with eight to ten strokes in a small (7 ml) glass Dounce homogenizer (Kontes Co., Vineland, N. J.). The microsomes were then concentrated by sedimentation, the supernate was removed, and the soft pellet was thoroughly mixed with a glass micropipette. Aliquots of the RM were then pipetted onto 3 mm cardboard discs and rapidly frozen in Freon 22 (Virginia Chemicals, Inc., Portsmouth, Mass.) cooled to -160°C by liquid nitrogen. For deep-etching, the microsomes were handled in an identical manner, but distilled water was substituted for the glycerol-TKM. Pieces of whole rat liver were minced with a razor blade in 2% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.4), and allowed to fix for 1 h at 4°C. After washing in buffer for at least 2 h, the samples were placed in 10% glycerol-TKM overnight at 4°C before freezing on cardboard discs.

Platinum-carbon replicas were made in a Balzers BAF 301 apparatus (Balzers AG, Balzers, Liechtenstein) according to the procedures of Moor and Mühlethaler (38). The stage temperature was maintained at either -120°C for fracturing or -112°C in experiments which required etching. Although most investigators use -100°C for deep-etching, we have found that, in general, 30–45 s of etching at -112°C was sufficient to expose microsomal surfaces. Higher temperatures, or longer etching times, frequently led to extensive replica fragmentation. Replicas were cleaned by floating on a commercial bleach solution for 1 h, washed with distilled H₂O and mounted on uncoated 400-mesh grids. The terminology proposed by Branton et al. (13) was adopted for identification of the fracture faces and membrane surfaces.

Quantitation of Intramembrane

Particle Size and Density

Electron micrographs of freeze-fractured microsomes

taken at $\times 24,000$ were enlarged photographically to $\times 141,000$. Areas of relatively flat individual microsomal fracture faces were measured with a compensating polar planimeter (model 620005, Keuffel & Esser Co., Morristown, N.J.), and the intramembrane particles within the measured area counted to give particle density. At least $1 \mu\text{m}^2$ of membrane area was measured for each of three rough microsomes preparations. Particle diameters of 250 particles for each fracture face were measured using a $\times 7$, magnifier equipped with a calibrated micrometer grating. In cases where particle boundaries were fuzzy or unclear, minimum particle diameters were recorded.

Sources

RNase A was obtained from Worthington Biochemical Corp. (Freehold, N. J.); T1 RNase from Calbiochem (San Diego, Calif.); T2 RNase, lysozyme, and polylysine from Sigma Chemical Co (St. Louis, Mo.); and puromycin from ICN Pharmaceuticals, Inc. (Life Sciences Group, Cleveland, Ohio).

RESULTS

Thin-section electron microscopy showed that the fraction of RM used in these experiments (Fig. 1) is homogeneous, consisting almost entirely of

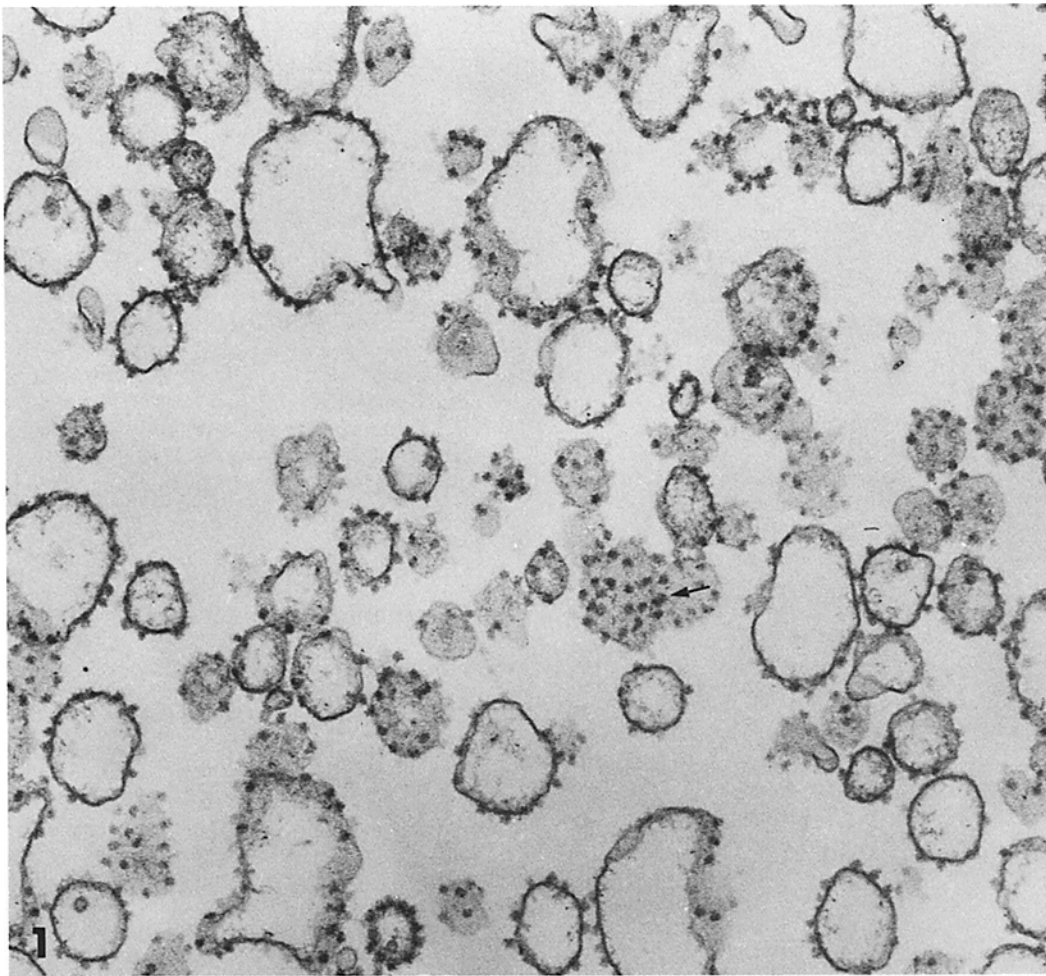


FIGURE 1 Representative field from a thin-section electron micrograph of a control preparation of RM washed in high salt and maintained at 24°C for 30 min before fixation. These microsomes contain only ribosomes tightly bound to the membranes through their nascent polypeptide chains. In vesicles sectioned tangentially to the membrane plane, patterns of membrane-bound polysomes can be observed (arrow). Average center-to-center distance between ribosomes within a polysome is $\sim 350 \text{ \AA}$, and the density is $\sim 390 \text{ ribosomes}/\mu\text{m}^2 \times 60,000$.

membrane vesicles (0.2–0.5 μm in diam) bearing numerous ribosomes (average diam $232 \pm 15 \text{ \AA}$) bound to their outer surfaces. In vesicles sectioned tangentially, groups of bound ribosomes frequently appeared to be arranged in polysomal patterns similar to those observed in intact cells (46, 47). The average center-to-center distance between individual ribosomes within a polysome was $345 \pm 36 \text{ \AA}$, and the overall density of ribosomes on the microsomal membranes was 391 ± 19 ribosomes/ μm^2 .

RM surfaces were also studied in replicas obtained from samples frozen in distilled water and then fractured and etched at -112°C to allow partial sublimation of the ice surrounding the frozen specimens (Fig. 2) and permit the examination of true membrane surfaces (51, 76, 77, 79). Most RM, however, were rarely intact after etching, and the fracture faces were extensively damaged, probably as a result of rapid water evaporation from the interior of the microsomal vesicles through the weakened half membranes (Figs. 3 and 4).

Membrane-bound ribosomes arranged in short polysomes were easily recognized in well preserved replicas of etched RM (Fig. 3). In general, their appearance was similar to that of bound ribosomes on the envelope of sea urchin nuclei described by Wartiovaara and Branton (79). Ribosomes bound to the surface of microsomes, however, were less tightly packed and, consequently, could be more clearly visualized. The average diameter of ribosomes in microsomal replicas ($262 \pm 20 \text{ \AA}$) was larger (by 30 \AA) than that observed in thin sections of RM. This difference could be attributed to the fact that images of thin-sectioned ribosomes are the result of heavy metal staining of dehydrated ribosomes, while the freeze-etch image is an image of rapidly frozen ribosomes with fully hydrated proteins. It is interesting to note that liver ribosomes viewed after negative staining (40), a procedure which utilizes air-drying, are of about the same diameter (230 \AA) as thin-sectioned ribosomes. Freeze-etched bound ribosomes appeared to be somewhat flattened (Figs. 3 and 4) and did not protrude as far from the membrane surface as they appear to do in thin sections (Fig. 1).

Occasionally, two regions were observed in deep-etched bound ribosomes which may correspond to the overlapping profiles of the two ribosomal subunits (Fig. 3). Such profiles would be expected in replicas containing top views of ribo-

somes bound to the membranes through their large subunits (66), since the small ribosomal subunits are narrower than the large (40). Microsomal surfaces in between ribosomes were generally smooth but, under optimal shadowing conditions, low irregular ridges which branched, or interconnected with one another, could be seen (Fig. 4). These ridges do not appear as distinct structures on the microsomal surface but, instead, may represent protuberances produced by underlying intramembrane structures. Similar ridges were also observed on the surface of microsomes from which ribosomes had been removed by the puromycin-HSB procedure (Fig. 5) and in areas of the microsomal surfaces which bear no ribosomes after RNase-induced ribosomal aggregation (Fig. 6, see following section for description of RNase experiments). Irregular prominences have also been described on the surface of erythrocyte membranes and attributed to the presence of intramembrane particles (52–54).

Although several studies have applied the freeze-fracture technique to RER membranes in fixed cells (44) and in isolated RM (17, 24, 33), only one report, that on the nuclear envelope of sea urchin embryos (79), appears to be available in which deep-etching was utilized to expose membrane-bound ribosomes. The difficulties in visualizing bound ribosomes are probably due to the fragility of microsomal membranes, which, like chloroplast membranes (45), seem to be severely damaged during etching by the sublimation of water through the fractured membranes. In contrast, plasma membranes are relatively unaffected (51, 76, 77), even after long periods of etching (27, 50). Our success in demonstrating bound ribosomes on the surface of isolated RM is probably due to both the short periods of etching (30–45 s) and the lower temperatures (-112°C) employed in these studies.

Mobility of Bound Ribosomes

The mobility of ribosomes and their binding sites on ER membranes was assessed in RM treated with RNase to cleave the mRNA of bound polysomes and allow for the potential randomization of individual ribosomes. RM were also incubated with antibodies against 60S ribosomal proteins in an attempt to produce ribosomal aggregates on the microsomal surface through the cross-linking of large subunits. After treatment at 4°C , control and experimental samples were either kept at 4°C or incubated further for 30 min at 24° or

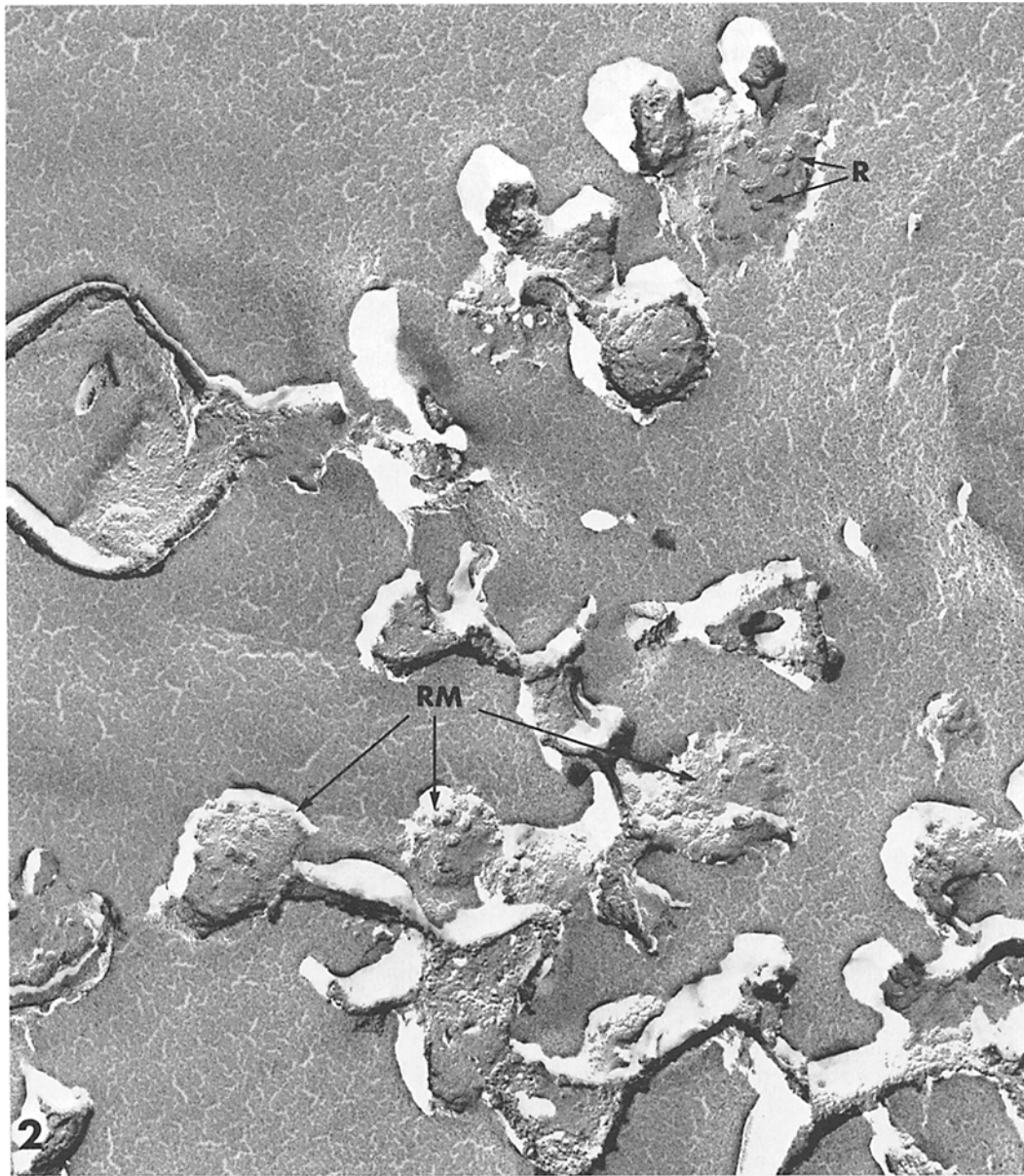


FIGURE 2 Freeze-etch electron micrograph of a replica obtained from control (*RM*) washed in HSB and maintained at 24°C for 30 min before fixation in glutaraldehyde and freezing in distilled water. After fracturing in a Balzers apparatus, the frozen microsomes were deep-etched for 45 s at -112°C. Membrane-bound ribosomes (*R*) can be clearly visualized on the microsomal surface as particles ~260 Å in diam. × 70,000.

37°C to increase the fluidity of the microsomal phospholipids (21, 56, 78).

Large aggregates of tightly packed bound ribosomes were apparent in both thin sections (Figs. 8-9) and deep-etched preparations (Fig. 10) of

RNase-treated *RM* incubated at 24°C. Thin sections of *RM* treated with RNase showed *RM* profiles with numerous membrane invaginations which were lined with aggregated bound ribosomes, while the remainder of the membrane sur-

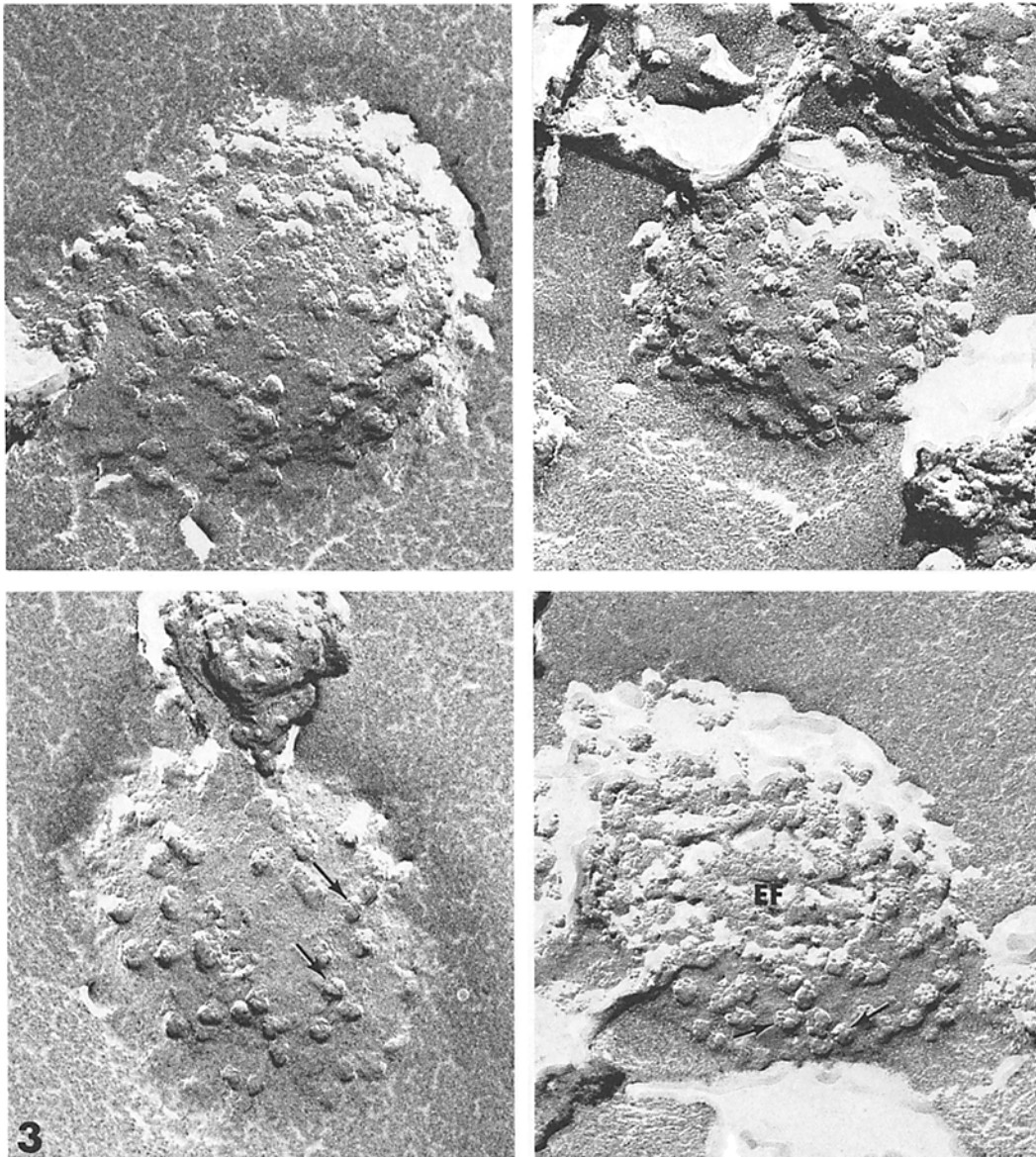


FIGURE 3 Selected examples of deep-etched RM. Bound ribosomes are not distributed at random on the microsomal surface and appear to form groups that correspond to the polysome patterns seen in thin sections. Ribosomal subunits can be observed in several ribosomes (arrows). Most of the well-preserved rough microsomal surfaces available for examination were those which had not been fractured before etching but, instead, lay just under the fracture plane. An endoplasmic fracture face (*EF*) which has been damaged by the etching process is shown in the lower right panel. In the upper panels, surface protuberances can occasionally be seen between the bound ribosomes (cf. Fig. 4). $\times 127,000$.

face was free of ribosomes (Figs. 8, 9*b*, and *d*). In other experiments (Figs. 9*c* and 10), however, it was apparent that extensive ribosome aggregation occurred without the formation of ribosome-lined membrane invaginations. It should be stressed

that, in all cases, after RNase treatment and incubation at 24° or 37°C, most bound ribosomes had moved laterally to produce aggregates and were confined to relatively small areas of the microsomal surface. Ribosome aggregation could also

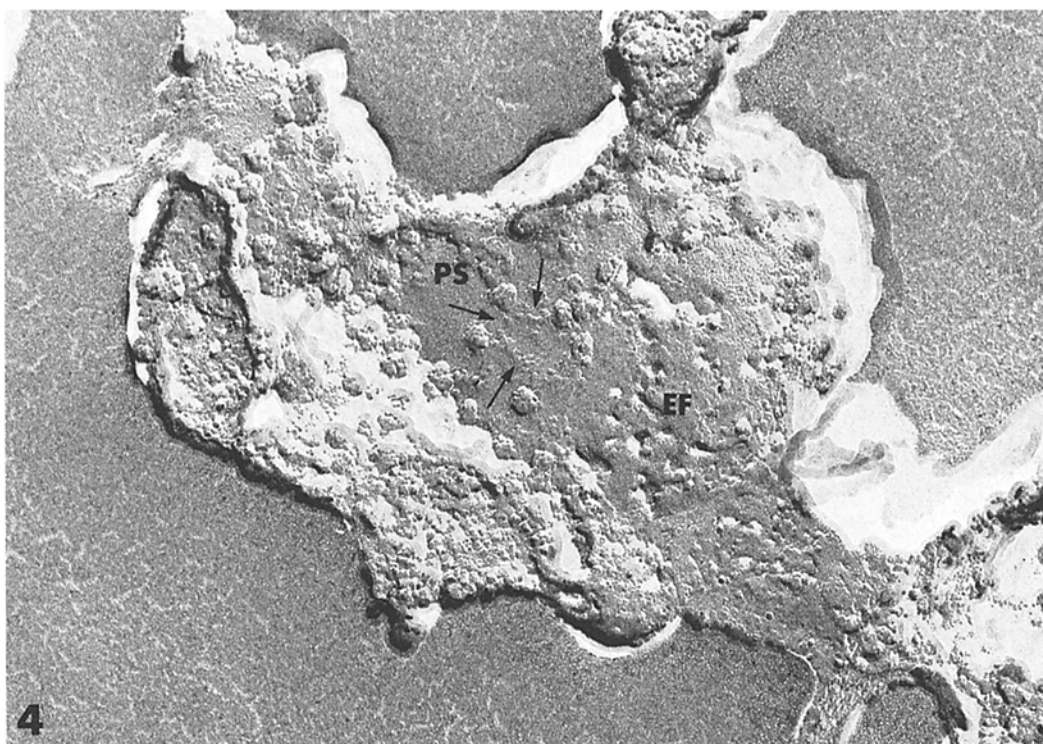


FIGURE 4 Deep-etched RM showing both the cytoplasmic surface (*PS*) and an endoplasmic fracture face (*EF*). Irregular surface protuberances, or ridges (arrows), extend in between the ribosomes. $\times 127,000$.

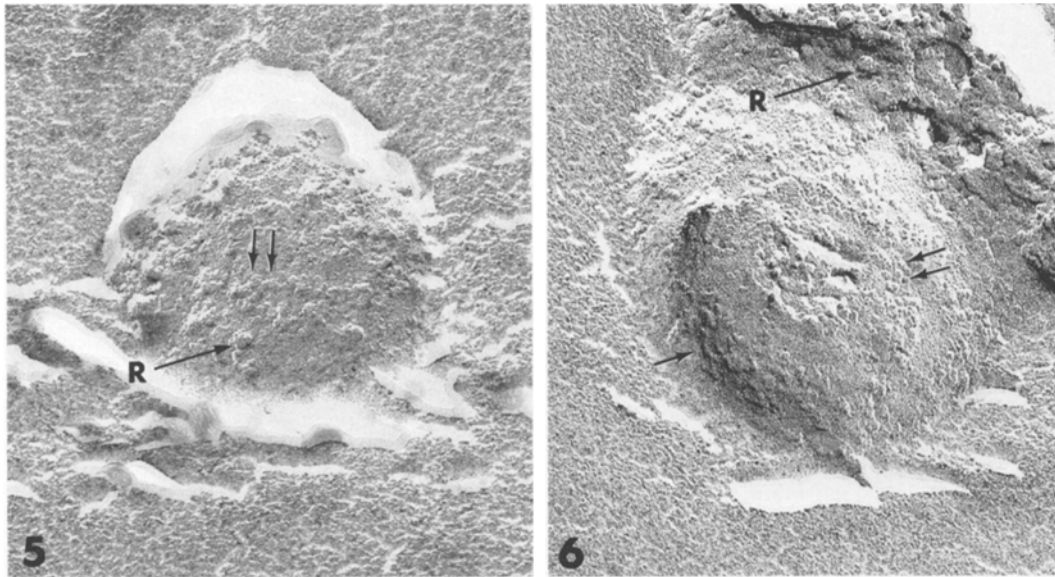
be achieved by incubation of RM in RNase for 15 min at 37°C, but the effects of temperature-dependent phase transitions could not be studied under these conditions.

Deep-etching of RNase-treated RM incubated at 24°C (Fig. 10) and 37°C (not shown) showed that the aggregated ribosomes were tightly packed in monolayers on limited areas of the microsomal surface. Boundaries of individual ribosomes within the aggregates were somewhat difficult to define but measurements of center-to-center distances for both deep-etched (~ 270 Å) and thin-sectioned (~ 240 Å) microsomes indicated that ribosomes of RNase-treated microsomes probably contacted one another.

Ribosomal aggregation was largely prevented when RNase-treated RM were maintained for 30 min at 4°C (Fig. 7); however, some small aggregates were still found during this incubation in the cold which often had the appearance of relatively straight rows of tightly packed ribosomes (Fig. 9*a*). Since these ribosomal aggregates are similar to, but shorter than, those observed on RNase-treated microsomes warmed to 24°C, they are

likely to be precursors of the larger ribosomal aggregates. This formation of small ribosomal patches preceding the appearance of larger aggregates is reminiscent of the process by which antibodies and lectins form "caps" on the surface of lymphocytes (15, 27, 75). Similar effects on ribosomal aggregation were also observed after treatment with either T1 or T2 RNase; however, large aggregates of bound ribosomes were not observed when RM were incubated at 24°C with inactivated RNase or cationic macromolecules such as polylysine or lysozyme. It should be noted that, after RNase treatment followed by incubation at 24°C, no significant release of ribosomes was observed by density gradient analysis in either TKM or HSB (Fig. 11*a* and *b*). However, RNase-treated ribosomes remained puromycin-dependent as shown by the release of ribosomal subunits (Fig. 11*c*). Our results (Fig. 11*a*) confirm previous findings by Blobel and Potter (8).

Ribosomal aggregation was also observed when RM were incubated with bivalent antibodies raised against proteins of bound 60S ribosomal subunits. After incubation in antibody for 15 min



FIGURES 5-6 Cytoplasmic surface features of rough microsomal membranes after ribosomal removal (Fig. 5) or aggregation of ribosomes after RNase treatment (Fig. 6). Ribosomes were removed by incubating RM in HSB containing 10^{-3} M puromycin for 30 min at 4°C followed by 30 min at 24°C . A bound ribosome (*R*) remaining on the surface is indicated by an arrow. Double arrows point to prominences or ridges on the membrane surface (Fig. 5). Ribosomes aggregated after ribonuclease treatment at 4°C followed by a 30-min incubation at 24°C are not visible on this region of the rough microsomal surface, and irregular prominences, or low ridges, are visible at both high (single arrow) and low (double arrows) shadow angles. $\times 127,000$.

at 4°C followed by 30 min at 24°C , extensive aggregation of bound ribosomes could be observed (Fig. 12). Since RNase was not used in these experiments, bound ribosomes were probably displaced as entire polysomes producing the large aggregates viewed in tangential sections (Fig. 12*a*). Microsomal aggregation due to the cross-linking of ribosomes from adjacent microsomes led to formation of large areas of ribosome-free membrane (Fig. 12*b*). The fact that large, tightly packed ribosomal aggregates are not observed after antibody treatment can be attributed to a lack of both rotational and translational freedom of individual ribosomes whose movements are constrained by the polysomal mRNA and by the imposition of antibody molecules which bind between the large ribosomal subunits. Incubation of RM in anti-60S antibody at 4°C , or antibody from control rabbits for 15 min at 4°C followed by 30 min at 24°C , did not produce ribosome aggregation.

Freeze-Fracture Observations on RER and RM

Preparations of fixed rat liver tissue and isolated

RM were studied after freeze-fracture in 10% glycerol in order to observe intramembrane features. In fractured hepatocytes, RER cisternae were easily recognized by their arrangement in parallel stacks (Fig. 13). Within a cisterna, the concave fracture face (*PF*) representing the cytoplasmic half of the ER membrane showed numerous intramembrane particles while the complementary luminal half of the membrane (*EF*) had few. Frequently, both membrane halves of the same RER cisterna could be visualized simultaneously when a fracture proceeding on one side of a large cisterna crossed the lumen to enter into the opposite side (Fig. 13). Our observations on freeze-fractured rat liver agree with those of Orci et al. (44).

Fracture faces in RM freeze-fractured in glycerol-TKM were morphologically similar to those of intact RER, indicating that gross membrane rearrangements do not take place during the isolation process (Fig. 14). The cytoplasmic fracture face (*PF*), which was more concave due to the increased curvature of the RM, contained the majority of intramembrane particles, $3,102 \pm 210$ particles/ μm^2 , while the convex luminal fracture

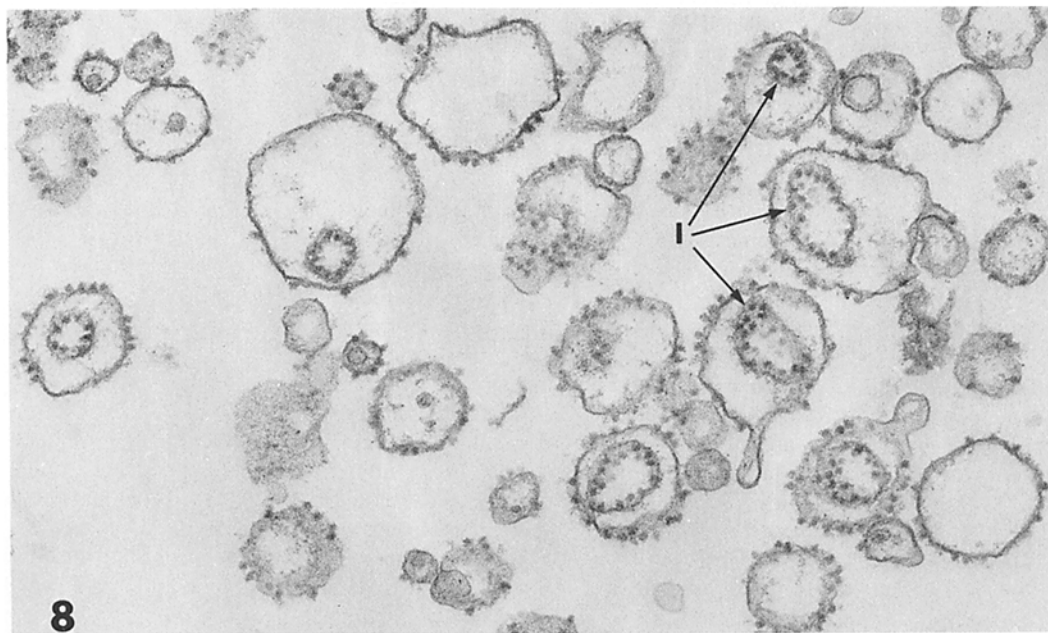
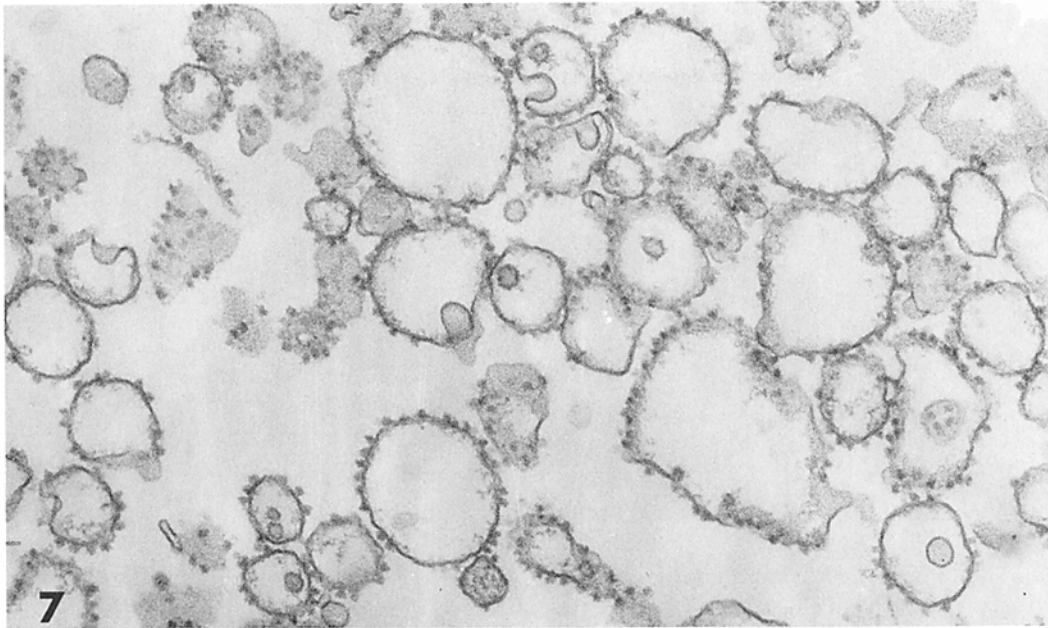


FIGURE 7 Rough microsomes incubated at 4°C for 15 min with pancreatic RNase (10 $\mu\text{g/ml}$), and then maintained at 4°C in TKM for a period of 30 min. The distribution of ribosomes in this preparation is similar to that in control RM (cf. Fig. 1) and appears unaffected by the RNase treatment. $\times 60,000$.

FIGURE 8 RM incubated in RNase (10 $\mu\text{g/ml}$ at 4°C for 15 min followed by incubation in TKM for 30 min at 24°C. Movement of ribosomes on the microsomal surface led to extensive ribosome aggregation. Areas of microsomal membrane bearing ribosome aggregates frequently invaginate into the lumen of the vesicles. In cross section these invaginations (*I*) appear as circular profiles with ribosomes lining the inner surface. $\times 60,000$.

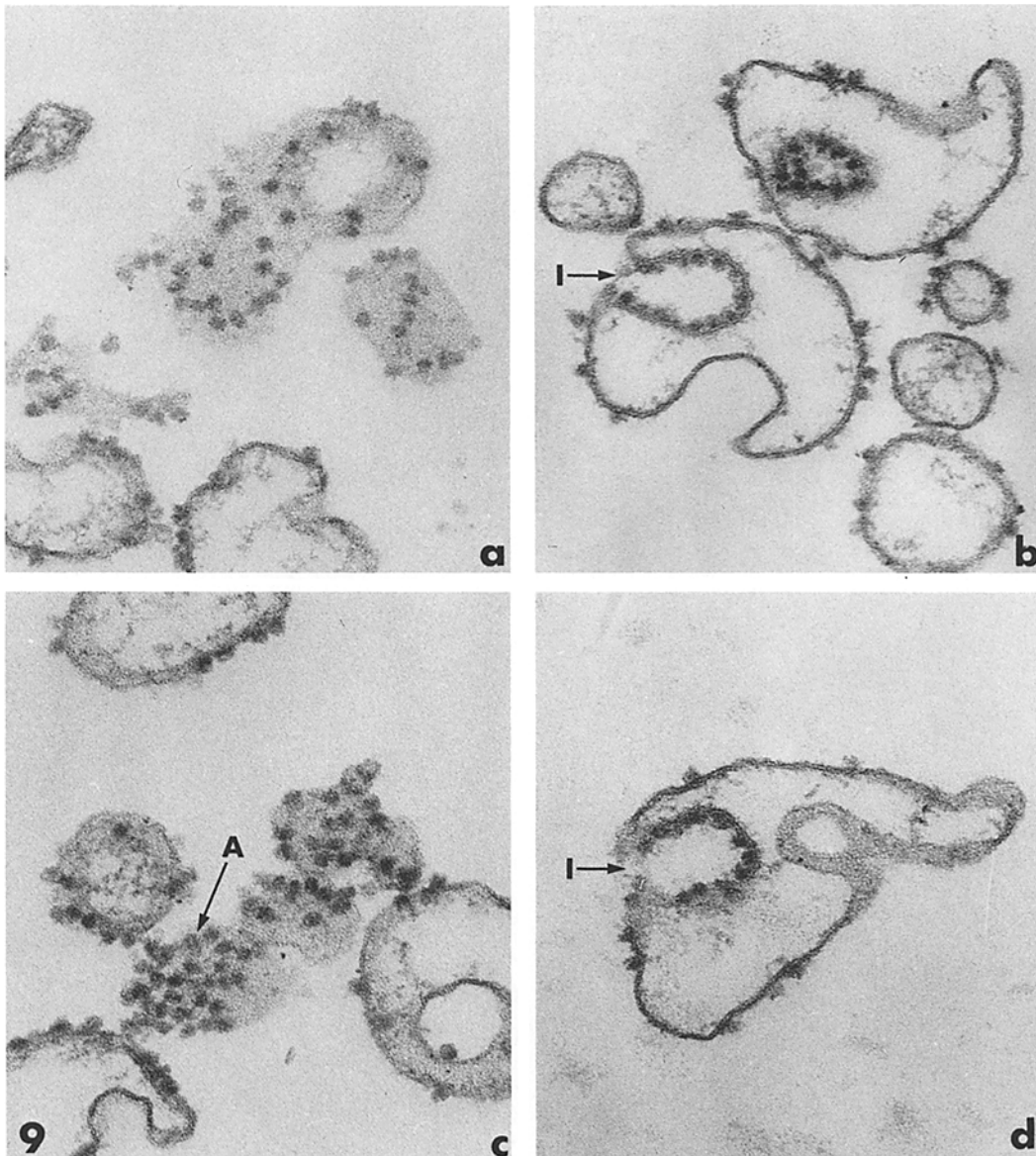


FIGURE 9 Gallery of selected electron micrographs from a preparation of RNase-treated RM. (a) RM maintained in TKM at 4°C after RNase. The ribosomes are homogeneously distributed, but some ribosomal patching may have occurred due to short range movement in the cold; (b-d), RM maintained in TKM at 24°C after RNase treatment. The membrane-bound ribosomes appear to have moved laterally over the microsomal surface into either membrane invaginations (*I*) or large aggregates (*A*). It should be noted that when ribosome-lined membrane invaginations were formed (*b* and *d*), the remainder of the ribosomal surface was ribosome-free. $\times 90,000$.

face (*EF*) had only 386 ± 58 particles/ μm^2 . The distribution of particle sizes was essentially the same for both fracture faces, with an average diam of 105 Å. Some particles on the *PF* face appeared

to be arranged in tightly packed interconnecting rows, but, in general, most were rather homogeneously distributed (Fig. 14).

Surface-bound ribosomes were never observed

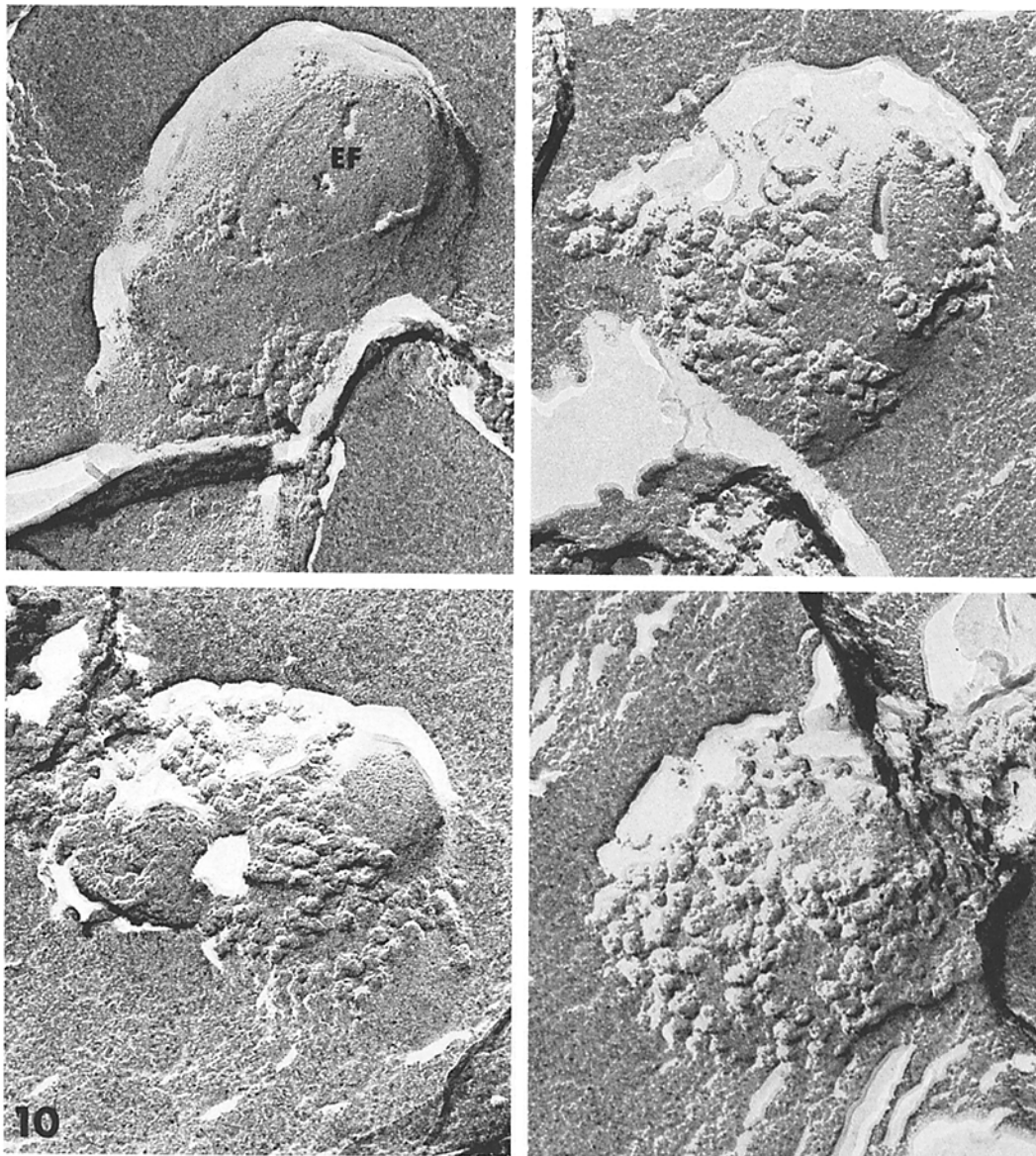


FIGURE 10 Extensive ribosome aggregates on the surface of freeze-etched RM after RNase treatment at 4°C followed by incubation in TKM at 24°C for 30 min. Large areas of ribosome-free membrane surface can be seen in these microsomes. The RM in the upper left panel was fractured before deep-etching. A short step from the fracture face (*EF*) up to the microsomal surface with its aggregated ribosomes can be clearly visualized. $\times 127,000$.

after fracturing RM in glycerol and replicating without etching (Figs. 14 and 15). Only when etching was allowed to occur after fracturing in H₂O could bound ribosomes be observed on the surface of the microsomes simultaneously with an *EF* face (Figs. 3, 4, and 10). These observations

indicate, that, during freeze-fracture, rough microsomal membranes split along an interior plane as proposed by Branton (12). They contradict a recent report by Hochberg et al. (24) who identified the 90–110 Å particles seen in freeze-fracture RM preparations as surface-bound ribo-

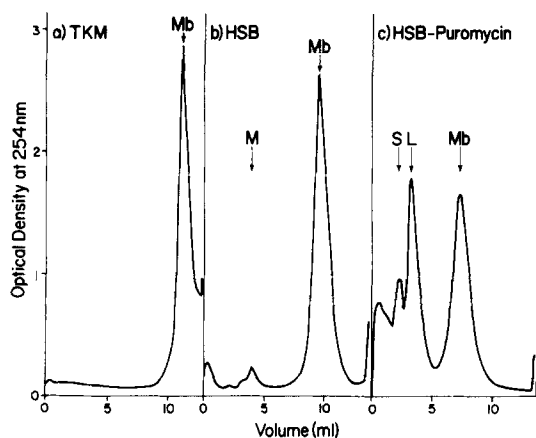


FIGURE 11 Effects of puromycin on RNase-treated RM. After RNase treatment, as described in the text, the RM were adjusted to the appropriate salt concentrations (a) TKM, or (b and c) HSB, 2.5 mM $MgCl_2$, with (c) or without (b) 10^{-3} M puromycin. All samples were then incubated for 30 min at $4^\circ C$ and 30 min at $24^\circ C$. Samples (~ 10 OD units at 260 nm) were loaded onto 12.5 ml of a 10–55% linear sucrose gradient containing TKM (a) or HSB (b and c) and centrifuged for 2 h at 40,000 rpm in the SW41 rotor at $4^\circ C$. The optical density distribution at 254 nm was measured in a Uvi-cord monitor equipped with a log converter by withdrawing the gradients from the top of the centrifuge tubes with a Buchler Auto Densiflow probe (Buchler Instruments, Inc., Fort Lee, N. J.). Almost no ribosomes were released from RNase-treated RM sedimented in (a) TKM or (b) HSB; however, the ribosomes were still responsive to puromycin treatment after RNase and were extensively removed from the membranes (c).

somes and, subsequently, concluded that microsomes do not fracture down an internal hydrophobic plane.

Changes in the Distribution of Intramembrane Particles

Freeze-fracture studies were also carried out to investigate possible relationships between the ribosomes bound to the microsomal surface and the intramembrane particles. Complete removal of the ribosomes from the RM membranes by incubation in puromycin-HSB led to extensive aggregation of the particles, concomitantly producing large membrane regions free of particles (Fig. 15). Similar rearrangements of intramembrane particles were also observed in RM incubated at $24^\circ C$ after RNase treatment to produce ribosome aggregation. On the other hand, treatments which did not lead to removal, or aggregation, of ribosomes,

such as incubation with puromycin in TKM, incubation in HSB without puromycin, or maintenance of RNase-treated RM at $4^\circ C$, did not produce intramembrane particle aggregation.

DISCUSSION

The observations reported in the preceding sections demonstrate that, under experimental conditions, bound ribosomes can exhibit extensive lateral displacement on the surface of isolated RER membranes. We found that two-dimensional ribosomal aggregates consisting of monolayers of packed ribosomes were formed on the surface of RM when these membranes were maintained at 24° or $37^\circ C$ after treatment with RNase or during incubation at $24^\circ C$ with antibodies against proteins of the large ribosomal subunits.

It has recently been reported (34, 35) that free ribosome monomers can also form large aggregates when incubated at $37^\circ C$ in the presence of microsomes or in postmitochondrial supernate. These aggregates, which may result from the activation of endogenous ribonucleases, were reported to be capable of forming an artefactual association with microsomal membranes (34, 35). In our experiments, however, several observations demonstrate that aggregates formed by membrane-bound ribosomes were not due to the deposition of ribosomes which had been previously detached from the membranes during incubation in RNase. Highly purified fractions of RM which were previously washed in media of high salt concentration and, therefore, contained only ribosomes firmly anchored to the membranes by their nascent polypeptides (2) were used in our studies. Moreover, sucrose density gradient analysis demonstrated that, under the conditions of incubation with RNase, release of ribosomes from the microsomal membranes was negligible.

The unaltered appearance of ribosomes from RNase-treated RM suggested that, in spite of the degradation of exposed mRNA and rRNA, the integrity of the bound ribosomes was not greatly affected by the nuclease treatment. We found that, in agreement with previous observations on RNase-treated ribosomes (14, 23, 31), an intact rRNA was not necessary for peptide bond synthesis since ribosomes aggregated on RNase-treated RM remained functionally capable of coupling puromycin to their nascent polypeptides. This led to an extensive puromycin-dependent release of ribosomal subunits when microsomes containing ribosomal aggregates were analyzed by density

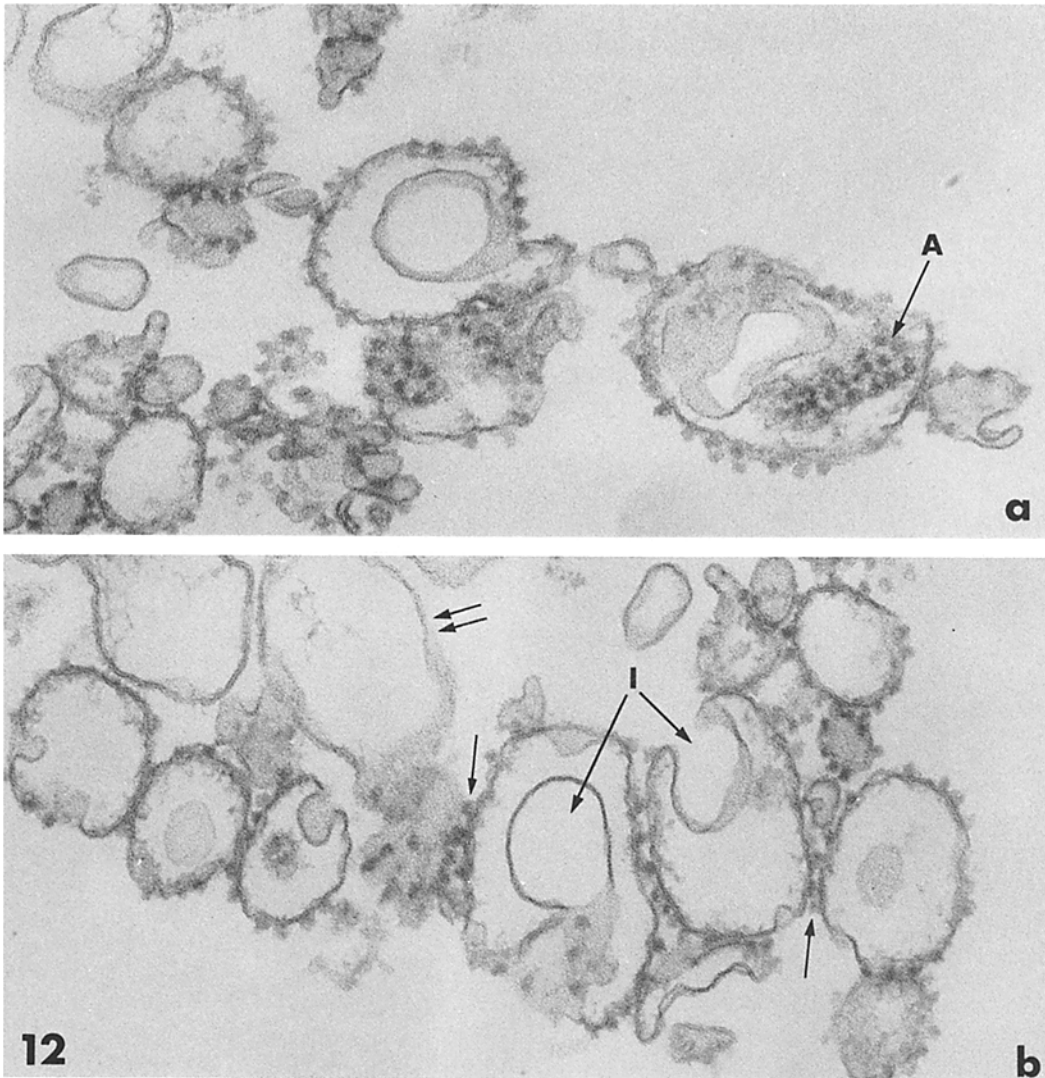


FIGURE 12 Aggregation of bound polyribosomes on RM treated with antibodies against 60S ribosomal proteins. RM were incubated with the antibody preparations ($170 \mu\text{g}$ protein/ml in TKM) for 15 min at 4°C , followed by 30 min at 24°C . These microsomes were not treated with RNase, and ribosome aggregation (A) occurred as a result of the displacement of entire membrane-bound polysomes. In the upper micrograph (a), the large ribosome aggregate (A) visualized in tangential section was formed as a result of cross-linking large (60S) ribosomal subunits to one another. In addition, there is extensive cross-linking of ribosomes from adjacent membrane vesicles (b, single arrows). Such cross-linking immobilizes the ribosomes and allows the formation of large ribosome-free membrane areas (double arrows) many of which form membrane invaginations (I). $\times 95,000$.

gradient centrifugation. The release of ribosomes by puromycin-HSB indicates that, during the movement which leads to their aggregation, ribosomes are displaced together with their nascent polypeptide chains, membrane binding sites, and other associated membrane components necessary

for vectorial discharge of newly synthesized polypeptides.

Although the architectural details of ribosome binding sites have yet to be elucidated, previous studies (11) strongly suggest that proteins are important components of the binding sites. In addition,

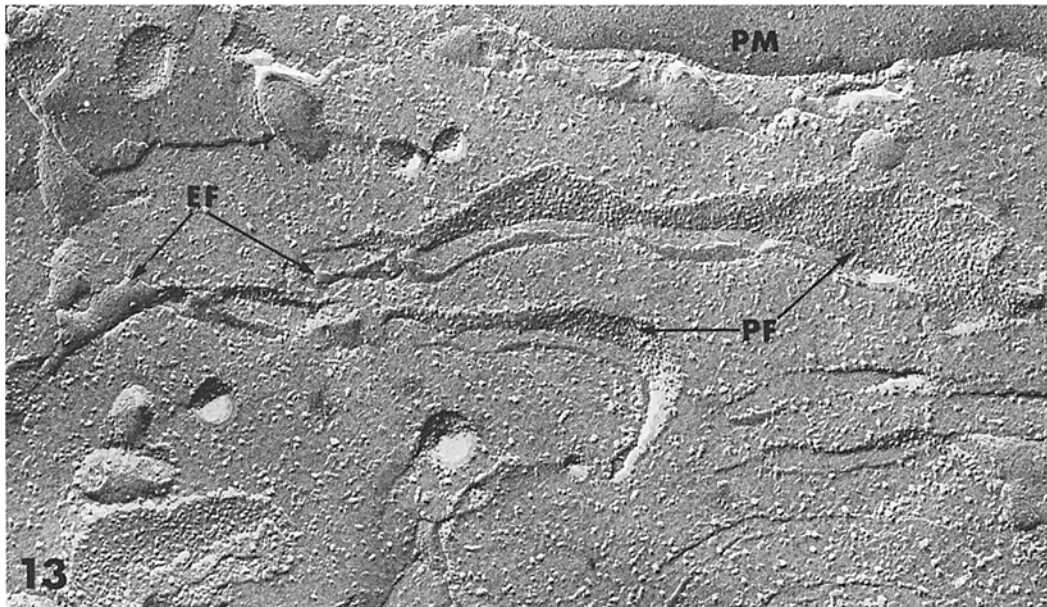


FIGURE 13 Freeze-fracture preparations of intact rat liver. Several cisternae of the RER can be seen with the cytoplasmic fracture faces (*PF*) containing the majority of intramembrane particles while the complementary *EF* face has few. A portion of the plasma membrane (*PM*) can also be observed. $\times 64,000$.

tion, since the binding capacity of RM stripped of ribosomes is unaffected by either high salt or EDTA treatment, it can also be presumed that proteins of the binding site are intrinsic membrane components interacting directly with the microsomal phospholipid bilayer (64, 65). Recently, two proteins (mol wt 65,000 and 68,000) have been recognized in membranes derived from the RER which are absent in membranes derived from the smooth endoplasmic reticulum (SER) and in other cell membranes (29, 30, 65). These two proteins can be isolated together with the ribosomes after treatment of RM with neutral detergents in media of low ionic strength. Under these conditions, most other membrane components are removed and only a residual structure forming a proteinaceous network with ribosomes attached remains. Because these proteins have been cross-linked to the bound ribosomes with reversible cross-linking reagents (29), they are thought to be components of the binding sites. Constituents of the binding sites are probably organized into functional complexes responsible for accepting and processing selected classes of polypeptides manufactured in bound polysomes. It can therefore be expected that a multitude of functions are associated with the ribosome binding sites and the ribosome-membrane junctions. These in-

clude: (a) binding of large ribosomal subunits (66), (b) the recognition and cleavage of the hydrophobic signal peptide in nascent polypeptide chains (5, 6, 10, 16), (c) the transfer of nascent polypeptides across the RER membranes (58, 59), and (d) posttranslational modifications necessary to determine the final destination of membrane polypeptides (39, 64). Because the proteins implicated in ribosome binding are quite large (29), it appears likely that ribosome binding sites and any associated functional complexes could be represented by intramembrane particles. Considerable evidence indicates that, in other membranes, intramembrane particles contain proteins which have a transmembrane disposition (52-54, 77). Since, in the RM there are considerably more intramembrane particles than bound ribosomes (10:1), it is reasonable to conclude that only a small fraction of the particles could have a direct association with the ribosomes. We found, however, that, during RNase-induced ribosome aggregation, a major change in the overall distribution of the intramembrane particle population occurred, but we were unable to directly correlate the redistribution of the aggregated ribosomes with that of the aggregated intramembrane particles on the *PF* face. This was due to the fact that bound ribosomes and the *PF* face cannot be visual-

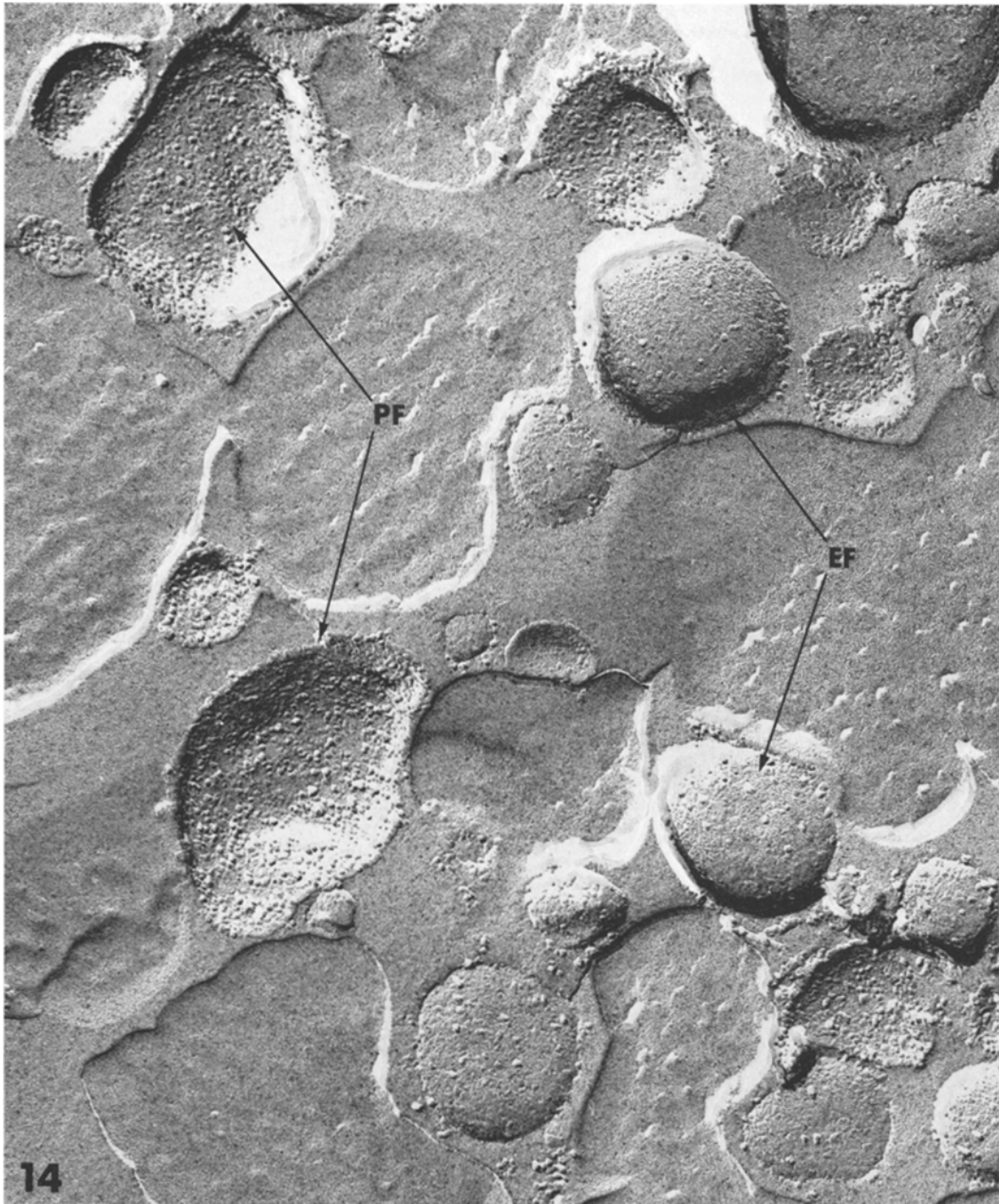


FIGURE 14 Freeze-fracture electron micrograph of RM washed in HSB and maintained at 24°C for 30 min before freeze-fracture. Although some intramembrane particles appear to be arranged into short rows, most particles on the concave cytoplasmic fracture face (*PF*) are homogeneously distributed with a density of $\sim 3,200$ particles/ μm^2 . The density of particles on the concave luminal fracture face (*EF*) is ~ 400 particles/ μm^2 . These microsomes have not been treated with RNase. $\times 95,000$.

ized simultaneously on the same microsomal vesicle. Nevertheless, it appears unlikely that particle aggregation occurred only in areas of ribosome aggregation, or vice versa, since aggregated ribo-

somes seemed to be concentrated in relatively small regions of the microsomal surface while particle redistribution was observed throughout the fractured membranes. Moreover, the redistribu-

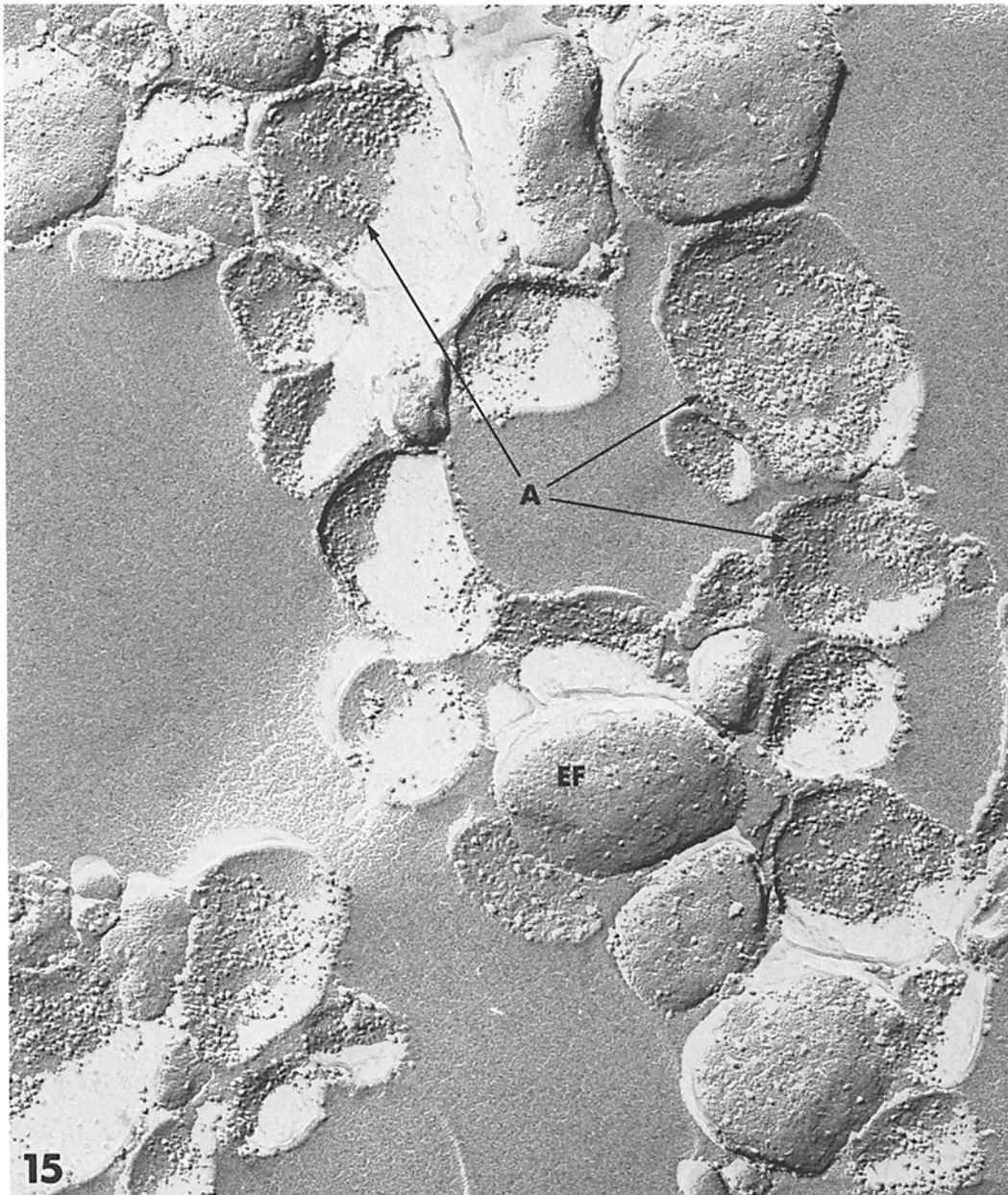


FIGURE 15 Redistribution of intramembrane particles in RM from which bound ribosomes were removed by incubation for 30 min at 24°C in HSB containing 10^{-3} M puromycin. The intramembrane particles on the PF face have formed aggregates (A), leaving intervening particle-free regions. A similar intramembrane particle aggregation occurs in RM after ribosomes are aggregated by RNase treatment at 4°C followed by incubation for 30 min at 24°C. $\times 95,000$.

tion of particles which occurred after ribosome removal was indistinguishable from that which occurred after the ribosome aggregation. Although protuberances on the cytoplasmic surface of mi-

croosomes stripped of ribosomes were detected, none of these could be definitively identified as part of the ribosome binding sites. Indeed, our observations on the redistribution of particles after

ribosome removal provide no information on the distribution of binding sites on membranes stripped of ribosomes. Since electron microscope observations (not shown) demonstrate that ribosomes rebound to stripped membranes are homogeneously distributed over the microsomal surface, it is possible that (a) after ribosome detachment, binding sites were not aggregated or (b) the aggregation of binding sites is reversed by ribosome rebinding. Therefore, it appears probable that the redistribution of the intramembrane particles after ribosome aggregation, or detachment, reflects a reorganization of membrane components much more extensive than the one which may affect just the binding sites. The displacement of the membrane proteins associated with the ribosome binding sites (29, 30, 65) may have a considerable effect on the overall membrane organization causing particle redistribution. In this respect, it should be noted that rearrangement of ribosomes has also been observed on the surface of RM treated with low concentrations of the same neutral detergents which, at high concentrations, are used for the isolation of the proteinaceous network linking the binding sites (30). That an extensive displacement of membrane components occurs concomitant with the ribosome movement is also suggested by the changes in curvature and appearance of the microsomal membranes at the site of ribosome aggregation. Although changes in curvature and invaginations could simply be the result of the close packing of aggregated bound ribosomes, they may also reflect structural specializations in some regions of the ER membranes, which become morphologically apparent. Thus, areas of the ER which bear ribosomes are usually organized into flattened, stacked cisternae while smooth areas of the ER are tubular and more convoluted. These variations in membrane morphology may reveal the presence, or absence, of the proteinaceous network which is associated with the binding sites.

Although the mechanism by which bound ribosomes are displaced on the rough microsomal membranes remains unknown, the observation that incubation in the cold prevents ribosome aggregation suggests that the fluidity of the microsomal lipid bilayer is a necessary requirement for ribosome movement. A thermotropic phase transition which affects the fluidity of the membranes has been observed by other investigators (21, 56, 78), who detected temperature-dependent changes in the microsomal phospholipids within

the 19°–22°C range. Both cytochrome b_5 and the flavoprotein NADH cytochrome b_5 reductase (60, 61, 74, 81) appear to be capable of random diffusion in the plane of rough microsomal membranes, and the function of both monooxygenase (18) and glucuronyltransferase (82) is clearly affected by temperature changes controlling the rigidity of the membrane phospholipids. The possibility should also be considered that a phase separation of membrane proteins and lipids (69) affects the mobility of ribosomes in the rough microsomal membranes. Our results show that, above the transition temperature, ribosomes, their associated binding sites and nascent polypeptides and, possibly, other associated membrane structures can be displaced by lateral diffusion in the plane of a fluid RER membrane. We propose that there is constant motion of the ribosomes on the microsomal surface at physiological temperatures. Cleavage of the mRNA with RNase would allow an increase in their freedom of movement and in the frequency of ribosomal collisions. The freely mobile ribosomes, due to damage of their rRNA, become "sticky" and adhere to one another forming, at first, small clusters, which eventually become larger aggregates. The observations on the redistribution of ER membrane components reported in this paper are in agreement with those of other investigators (17, 18, 21, 56, 60, 61, 74, 78, 81, 82) and are in accord with the predictions of the fluid mosaic model proposed for membrane structure (19, 71, 72).

Recently, Duppel and Dahl (17) have used freeze-fracture electron microscopy to study the effects of temperature-dependent phase transitions in total microsomes (both rough and smooth) derived from the liver of rats which received phenobarbital, a treatment leading to proliferation of the SER. These investigators reported that, after incubation of total microsomes at 4°C, intramembrane particles undergo aggregation. The subcellular origin of the membranes containing aggregated particles, however, was not determined, and it is possible that the aggregation observed by Duppel and Dahl occurred in membranes other than those of RM. A temperature-dependent aggregation of intramembrane particles in outer mitochondrial membranes, a common contaminant of total microsome fractions, has been observed by Höchli and Hackenbrock (25). In addition, we have not observed particle aggregation in purified RM maintained at 4°C.

It remains to be determined whether factors

other than ribosome aggregation or ribosome removal can affect the distribution of intramembrane particles in RER membranes and whether such changes have physiological importance. In other systems, particle mobility has been observed accompanying such biological events as secretion in *Tetrahymena* (67), water transport in toad bladder (26), and ion-induced stacking of chloroplast membranes (37, 43).

It should be noted that, although our observations serve to emphasize the potential mobility of the ribosome binding sites in microsomal membranes, we have provided no direct evidence indicating that membrane-bound ribosomes are mobile during protein synthesis *in vivo*. In principle, the relative movement of ribosomes with respect to mRNA necessary for translation may be accomplished by movement of ribosomes and their associated binding sites in the plane of the membrane as well as by displacements of the mRNA on immobilized ribosomes. Although, in some systems, a direct association of the mRNA with the membranes through a segment located near the 3' end of the messenger (3, 32, 36) may also restrict the mobility of the mRNA molecule, it has been noted (64, 65) that a structural arrangement may be envisaged which would allow translation to proceed even if both ribosomes and mRNA are bound to the membrane and fixed relative to one another. This would require the existence of a long untranslated segment in the mRNA molecule towards its point of attachment to the membrane and could lead to polysomal patterns observed similar to those observed on RER membranes by electron microscopy (4, 46, 47).

On the other hand, it has been observed that Arrhenius plots of protein synthesis activity in RM show a discontinuity coinciding with the melting temperature of microsomal phospholipids (78). This observation would be consistent with the notion that, during translation on bound ribosomes, decoding of mRNA is facilitated by the displacement of ribosomes and their associated binding sites in the plane of the rough microsomal membrane. An increased rigidity in the phospholipid bilayer would decrease ribosome mobility, slow down messenger decoding and account for the higher activation energy for protein synthesis below the phospholipid transition temperature.

Characteristic spiral patterns thought to represent polysome configurations have been observed with an electron microscope in grazing sections of RER membranes (4, 46, 47). The disappearance

of these patterns after inhibition of initiation of protein synthesis by Verrucaric acid or ethionine (3, 4) and the reappearance of the patterns upon recovery from the inhibition (4) provide evidence for the mobility of the binding sites *in vivo*. The ability of unoccupied ribosome binding sites to move in between rounds of translation could also effectively facilitate the assembly of bound polysomes by insuring the availability of binding sites for new initiation complexes near the free 5' end of mRNA's translated in association with membranes. If, indeed, ribosome binding sites are mobile *in vivo*, as the aforementioned observations suggest, the range of movement of ribosomes on the ER membrane should be limited to domains defined by the existence of the intramembrane proteinaceous network to which ribosomes are bound since binding sites are present only in rough regions of the ER (11),¹ in spite of the continuity of SER and RER membranes.

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