

PROTEINS OF ROUGH MICROSOMAL MEMBRANES RELATED TO RIBOSOME BINDING

I. Identification of Ribophorins I and II, Membrane Proteins Characteristic of Rough Microsomes

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ABSTRACT

Rat liver rough microsomes (RM) contain two integral membrane proteins which are not found in smooth microsomes (SM) and appear to be related to the presence of ribosome-binding sites. These proteins, of molecular weight 65,000 and 63,000, were designated ribophorins I and II, respectively. They were not released from the microsomal membranes by alkali or acid treatment, or when the ribosomes were detached by incubation with puromycin in a high salt medium. The anionic detergent sodium deoxycholate caused solubilization of the ribophorins, but neutral detergents led to their recovery with the sedimentable ribosomes. Ribosomal aggregates containing both ribophorins, but few other membrane proteins, were obtained from RM treated with the nonionic detergent Kryo EOB (2.5×10^{-2} M) in a low ionic strength medium. Sedimentation patterns produced by these aggregates resembled those of large polysomes but were not affected by RNase treatment. The aggregates, however, were dispersed by mild trypsinization ($10 \mu\text{g}$ trypsin for 30 min at 0°C), incubation with deoxycholate, or in a medium of high salt concentration. These treatments led to a concomitant degradation or release of the ribophorins. It was estimated, from the staining intensity of protein bands in acrylamide gels, that in the Kryo EOB aggregates there were one to two molecules of each ribophorin per ribosome. Sedimentable complexes without ribosomes containing both ribophorins could also be obtained by dissolving RM previously stripped of ribosomes by puromycin-KCl using cholate, a milder detergent than DOC. Electron microscope examination of the residue obtained from RM treated with Kryo EOB showed that the rapidly sedimenting polysome-like aggregates containing the ribophorins consisted of groups of tightly packed ribosomes which were associated with remnants of the microsomal membranes.

KEY WORDS rat liver · rough microsomes · membrane structure · membrane bound ribosomes · ribosome binding site

Membranes of the endoplasmic reticulum (ER) form an intracellular system of intercommunicating tubules and sacs which extends from the

perinuclear cisterna to the vicinity of the Golgi apparatus and the cell periphery.

Depending on the cell type, more or less extensive areas of the ER are found to bear ribosomes attached to the cytoplasmic face of the membranes (69, 71, 72). These ribosomes are engaged in the synthesis of secretory polypeptides and polypeptides to be incorporated in ER membranes and other subcellular organelles (70, 78). The binding of the ribosomes to the ER membranes occurs through specific sites on the large ribosomal subunits, near the place of exit of the nascent polypeptide chain (4, 16, 18, 77, 80).

Membrane sites providing for the binding of ribosomes to the ER membranes are of considerable functional interest. They insure that selected polysome classes discharge their products into the lumen of the ER cisternae. To accomplish this selective discharge, sites on the membranes must recognize specific classes of nascent polypeptides, and then interact with specific ribosomal proteins to effect the binding of the ribosome (79). This specific binding appears to facilitate the transfer of the polypeptides across the membranes and the cleavage of hydrophobic signals characteristic of secretory polypeptides (13, 14, 17, 59). Membrane components at or near the binding site must also carry out modifications of the nascent chains by glycosidation (81), hydroxylation (27), or cross-linking (25), which may serve to ensure the fate of the products.

A characterization of the structural elements which participate in the ribosome membrane junction is necessary to understand the function of bound ribosomes and the role of endoplasmic reticulum membranes in protein biosynthesis, secretion, and organelle biogenesis. Previous studies (see reference 76 for review) have suggested that sites for ribosome binding are characteristic of rough membranes (18, but see reference 85) and that proteins are important components of the binding sites, since mild proteolysis or heat treatment abolishes the *in vitro* ribosome binding capacity of membranes previously stripped of ribosomes (18, 41).

Morphological and biochemical observations (70, 78) indicate that sites for the binding of ribosomes are not uniformly distributed in ER membranes, but are segregated in specialized regions which normally bear ribosomes and hence are known as "rough" portions of the endoplasmic reticulum (ER). In their configuration and rela-

tionship to other organelles, membranes of rough portions of the ER are morphologically distinct from those without ribosomes. In hepatocytes and other secretory cells, for example, rough ER membranes are frequently arranged in stacks of several adjacent parallel cisternae while smooth ER membranes usually form tortuous or contorted tubules which branch, bend, or vesiculate frequently. Although it may be suspected that the presence of ribosomes underlies these morphological differences, a biochemical basis for the characteristic features of rough microsomal membranes has eluded previous investigations.

The experiments in this paper provide evidence for the presence in rough, but not in smooth microsomes, of two integral membrane proteins which are found in a constant stoichiometric ratio with respect to the ribosomes. These proteins, which were designated *ribophorins*, were isolated together with the bound polysomes, after solubilization of the membranes with the neutral detergent Kryo EOB. The presence of the ribophorins within ER membranes may explain the maintenance of the segregation of ribosomes within rough portions of the ER and may also account for the characteristic morphological appearance of rough ER cisternae. Preliminary reports of this work have been presented (45, 50, 79).

MATERIALS AND METHODS

General

All solutions were prepared in deionized glass-distilled water, filtered (0.45 μm or 1.2 μm for the concentrated sucrose and acrylamide solutions) and stored at 4°C. Centrifugations were performed in Beckman ultracentrifuges L3-50, L5-50, or L5-65 (Beckman Instruments, Spinco Div., Palo Alto, Calif.). The notation 30 min-30K-Ti60-4°C is used to specify centrifugation for 30 min in a Ti60 rotor at 30,000 rpm at 4°C. If not specified, centrifugation was done at 4°C. All pH determinations were made at room temperature.

Materials

Glycine, Trizma base (Tris), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, Mo.), *N,N'*-methylene-bisacrylamide, acrylamide, Triton X-100, and *N,N,N,N'*-tetramethyl ethylenediamine (TEMED), from Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, N.Y.); Coomassie brilliant blue and enzyme grade sucrose from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.); deoxycholic acid from Schwartz/Mann Div. and Aldrich Chemical Co. (Milwaukee, Wis.);

[methyl-³H]choline from New England Nuclear Corp. (Boston, Mass.); NCS solubilizer from Amersham Corp. (Arlington Heights, Ill.); puromycin dihydrochloride from Nutritional Biochemical Co. (Cleveland, Ohio); Ultrapure glutaraldehyde from Tousimis Research Corp. (Rockville, Md.); trypsin (3 × crystallized) and *N*- α -tosyl-L-lysyl chloromethyl ketone (TLCK) from Worthington Biochemical Corp. (Freehold, N.J.).

The nonionic detergent Kryo EOB (a polyethoxyalkylether) was a gift from Dr. D. H. Hughest (Miami Valley Research Laboratory, Proctor and Gamble Co., Cincinnati, Ohio).

Solutions

The following abbreviations were used: High salt buffer (HSB): 500 mM KCl, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂; HSB-10: 500 mM KCl, 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂; low salt buffer (LSB): 50 mM KCl, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂; TKM: 50 mM Tris-HCl pH 7.5, 25 mM KCl, 5 mM MgCl₂; 0.25 M-STKM: TKM containing 0.25 M sucrose; 2.0 M-STKM: TKM containing 2 M sucrose; 2 × TKM: 100 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂; 10% SLSB: low salt buffer containing 10% sucrose; 60% SLSB: low salt buffer containing 60% sucrose; 10% SHSB: high salt buffer containing 10% sucrose; and 25% SHSB: high salt buffer containing 25% sucrose. The notation LSB-TEA indicates that Tris was replaced by triethanolamine.

Cell Fractionation

Male Sprague-Dawley rats (150 g body weight) fasted for 18 h were used to prepare liver microsomes according to the procedure of Adelman et al. (3) or a modification which yields a cleaner preparation of rough microsomes (RM) although with smaller yield (51). In this modification, the postnuclear supernate is diluted with a half volume of water and then is centrifuged (20 min-17K-Ti60-4°C) at a slightly higher speed (17,000 rpm) than indicated by Adelman et al. (3). Washing of the mitochondrial pellets by recentrifugation is omitted. The postmitochondrial supernate (PMS) is then adjusted to 1.35 M sucrose and layered over a step gradient consisting of 5 ml of 2.0 M STKM and 5 ml of 1.55 M STKM containing rat liver high speed supernate as a source of RNase inhibitor (15). This gradient is overlaid with 1 M sucrose and centrifuged (16 h-40K-Ti60-3°C). RM were collected from the 1.55–2.0 M interface while smooth microsomes (SM) were collected from the 1.0–1.35 M interface by aspiration with a syringe through a bent 14-gauge needle. Both fractions were diluted with 1 vol of 2 × TKM, and the microsomes were recovered by sedimentation (15 min-35K-Ti60). Microsomes were resuspended in 0.25 M STKM; aliquots (~15–20 mg protein) were diluted 1:2 with glycerol and stored at –70°C. Unless otherwise noted,

before use microsomal suspensions containing glycerol were diluted (1:1) with 2 × HSB-10, and the microsomes were collected by sedimentation (20 min-30K-Ti60).

Sucrose Density Gradient Centrifugation

Composition of the gradients and conditions of centrifugation are given in the individual figure legends. To monitor the absorbance profiles throughout the gradients, these were collected by means of an auto densi-flow probe (Buchler Instruments Div., Searle Diagnostics, Inc., Fort Lee, N.J.) connected to an LKB uvicord II monitor and a perpex peristaltic pump (LKB Produkter, Stockholm, Sweden). A log converter connected to a Hewlett-Packard linear recorder (Hewlett-Packard Co., Palo Alto, Calif.) was used to obtain absorbance profiles at 254 nm. The radioactivity distribution was measured in fractions collected from the effluent of the system at time intervals. Aliquots (100 μ l) taken from each fraction and from the pellets resuspended in 0.5 ml of water were placed in mini vials (A-G Onics Corp., Brooklyn, N.Y.), incubated with 500 μ l of NCS (Amersham Corp.) at 60° for 60 min and counted with 5 ml of toluene scintillator (Liquifluor, New England Nuclear, Boston, Mass.) in LS 230 or LS 250 liquid scintillation counters (Beckman Instruments, Inc., Spinco Div.).

Detergent Treatment

Whenever possible, freshly prepared microsomes were used for extraction of membrane proteins with Kryo EOB since RM stored at –70°C gave less satisfactory results. Microsomes brought to HSB-10 were washed by centrifugation (20 min-30K-Ti60) and resuspended in LSB (3.5 mg protein/ml) using a hand-operated Potter-Elvehjem homogenizer. Detergent solutions at 10 times the desired final concentration were added to RM suspensions kept at 4°C while stirring with a vortex mixer. The incubation mixtures were kept for 30 min at 4°C before analysis on sucrose density gradients or subfractionation by differential centrifugation. In the latter case, 0.5–2 ml of a detergent-treated microsomal suspension was placed in an Oak Ridge centrifuge tube, underlayered with 0.3–2 ml of 20% SLSB containing detergent, and centrifuged (60 min-40K-Ti50 or Ti60). Supernates including the cushion were removed, and pellets were resuspended in an equivalent volume of distilled water. Aliquots (400 μ l) were prepared for electrophoresis according to Maizel (51).

Ribosome Removal

For puromycin treatment (4), RM washed in HSB-10 were resuspended in LSB. The final ion concentration was adjusted to HSB-2.5, with a compensating buffer. Puromycin (10⁻³ M) was added, and the suspension was kept for 20 min at 20°C and for 10 min at 37°C. After

dilution (4 times) with HSB, the membranes stripped of ribosomes (RMstr) were recovered by sedimentation (30 min-40K-Ti60) through a sucrose cushion (4 ml of 20% SHSB). For electrophoretic analysis, RMstr were resuspended in water. Aliquots of the RM suspensions incubated with puromycin were also analyzed in 10–25% SHSB density gradients under conditions (90 min-40K-SW41–20°C) which displayed the released ribosomal subunits.

In Vivo Labeling of RM Components

[methyl-³H]choline (sp act 0.55 Ci/mol) was administered intraperitoneally to rats 4 h before sacrifice to label microsomal phospholipids. Approx. 0.48% of the total injected radioactivity was recovered in the RM fraction which contained 5.2×10^4 dpm/mg of microsomal protein.

Polyacrylamide Gel Electrophoresis

Discontinuous SDS-acrylamide gradient gel electrophoresis was carried out in slab gels (resolving gel: 1-mm thick; 16 × 20 cm; sample gel: 3 × 20 cm) using a vertical electrophoresis apparatus similar to the one described by Studier (90). Slot formers provided 13 or 19 places for sample loading. In the resolving gel, an essentially linear acrylamide gradient was generated using a gradient mixing apparatus (Kontes Glass Co., Vineland, N.J.) connected to an LKB vario perpex pump (LKB Produkter) or, when several gradient gels were prepared simultaneously, to a 4-channel peristaltic pump (Sage Instruments Div., Orion Research Inc., Cambridge, Mass.).

Aside from minor modifications, all buffers and solutions were those described by Maizel (58). To stabilize the acrylamide gradients, sucrose (20% wt/vol) was added to the buffer containing the higher acrylamide concentration. The catalyst (ammonium persulfate) concentration was reduced to 0.125% to prevent polymerization during the formation of the acrylamide gradient. Samples for electrophoresis (up to 300 µg each) containing 20% glycerol, sample buffer, and 2% mercaptoethanol were heated to 90°C for 2 min and loaded into the sample slots by underlayering through the electrode buffer.

Electrophoresis in a Tris-glycine buffer (pH 8.9) was carried out at 15–25 mA until the tracking dye was half an inch above the bottom of the gel (~16 h). Gels were stained in 0.2% Coomassie brilliant blue dissolved in 50% methanol containing 7% acetic acid and destained in 30% methanol with 7% acetic acid. Stained gels were photographed through an orange filter using Kodak Ektapan film. Densitometric tracings of stained gels were made at 550 nm using a Gilford 240 spectrophotometer equipped with a scanning module and recorder (Gilford Instrument Co., Oberlin, Ohio). Myosin (210,000); β-galactosidase (130,000); serum albumin

(67,000); ovalbumin (45,000); urate oxidase (34,000); chymotrypsinogen (25,000); and globin (14,800) were used as molecular weight standards for the calibration of SDS-acrylamide gels.

Electron Microscopy

Microsome samples as well as subfractions obtained after detergent treatment were fixed for 30 min at 4°C, by adding an equal vol of 2% glutaraldehyde LSB-TEA. Fixed samples were either sedimented (10 min-10K-SW50) or collected on a Millipore filter according to Baudhuin et al. (8). Glutaraldehyde-fixed samples were washed overnight in 0.1 M cacodylate buffer pH 7.0, postfixed for 1 h in 1% OsO₄ in 0.1 M cacodylate, and stained with 1% uranyl acetate before dehydration and embedding in Epon 812. Thin sections were examined in a Philips 301 electron microscope operated at 80 kV.

Analytical Procedures

Protein was determined in duplicate aliquots according to the method of Lowry et al. (56) using bovine serum albumin (BSA) as a standard. The concentration of BSA was determined by OD at 279 nm using the procedure of Foster et al. (33). The RNA content of microsomes was estimated from the OD₂₆₀ of samples treated with 1% SDS (ribosome E₂₆₀^{1%} = 135; Tashiro and Siekevitz, 91) or determined by the procedure of Fleck and Munro (30). Phospholipids (PL) were extracted according to Folch et al. (32), and phosphorus was measured according to Ames and Dubin (6). For all determinations of optical density, a Zeiss PMQII spectrophotometer with cuvettes of 1-cm path length were used. Determinations of the cytochrome *b*₅, cytochrome P-450, and NADPH cytochrome *c* reductase content of microsomal fractions were made according to Omura and Sato (66) and Omura et al. (67), using an Aminco DW-2 spectrophotometer (American Instruments Co., Travenol Laboratories, Inc., Silver Spring, Md.).

RESULTS

Protein Composition of Rough and Smooth Microsomal Fractions

The most obvious difference between SDS-acrylamide gel electrophoresis patterns of rough and smooth rat liver microsomes is due to the presence of numerous bands corresponding to the ribosomal proteins in RM (cf. Fig. 1*a* with 1*c*). These proteins have, in general, molecular weights lower than 50,000 and are represented by bands located in the lower half of the electrophoretic gels. Gradient gels of high resolution (Fig. 1) also show, however, that, in addition to the ribosomal proteins, three other polypeptides (mol wt,

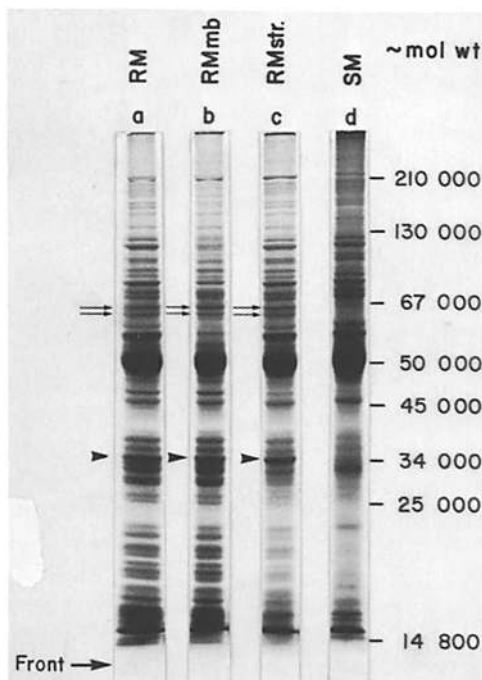


FIGURE 1 SDS-acrylamide gel patterns of proteins in rough and smooth microsomal fractions. Freshly isolated RM (a) and SM (d) were washed in LSB. A sample of RMstr (c) was prepared by removing ribosomes using the puromycin high salt procedure. RM depleted of luminal content (b) were obtained by treatment with a low concentration of DOC (1.3×10^{-3} M). Approx. 200 μ g of protein were loaded in the gel slot for SM. Other slots received an amount of protein equivalent to 250 μ g of RM. Analysis was carried out in SDS-acrylamide gradient gels (7–12%). Numbers on the right side indicate apparent molecular weights estimated from the mobility of marker proteins (see Materials and Methods) in a parallel track.

34,000, 63,000, and 65,000) are present in RM samples (arrowhead and arrows in Fig. 1), which are absent or represented only by very faint bands in gels of SM (Fig. 1d). These polypeptides are not of ribosomal origin: The intensity of the corresponding bands is not affected when ribosomes are removed from the membranes by treatment with puromycin in a high salt medium (RMstr), while bands representing ribosomal proteins disappear or greatly decrease in intensity after this treatment (cf. Fig. 1a with 1c). In fact, because of the removal of ribosomal proteins, other compositional differences between RM and SM are most evident when electrophoretic patterns of RM stripped of membranes (Fig. 1c) are compared with those of SM (Fig. 1d).

Polypeptides characteristic of RM are not extracted by treatment with low concentrations of DOC (Fig. 1b) or released by mechanical disruption procedures used to obtain the vesicular content of the microsomes (44, 47–49).

In addition to the differences between RM and SM due to proteins present exclusively in the former, a small number (3–4) of high molecular weight polypeptides, represented by minor bands in the upper third of the electrophoretic patterns (not marked in Fig. 1d, SM), was frequently present in SM but not in RM samples. Because SM fractions also contain fragments of mitochondria, Golgi apparatus, and plasma membranes, it cannot yet be ascertained whether these large molecular weight polypeptides are indeed components of smooth ER membranes. Contaminating membranes can, on the other hand, be largely eliminated from RM fractions, due to the higher density of the ribosome-studded vesicles.

The distribution of the polypeptides characteristic of RM subfractions obtained after treatment with high concentrations of the anionic detergent DOC, which is widely used to dissolve microsomal membranes, was also examined. After treatment of the RM with 1% DOC (2.5×10^{-2} M), only the polypeptide of mol wt 34,000 (indicated by an arrowhead in Fig. 2c) was recovered in the sedimentable subfraction which contains the ribosomes. The presence of the protein of mol wt 34,000 in gels of DOC-insoluble sediments containing mainly ribosomal proteins (Fig. 2c) in fact constituted the main difference between the set of proteins in this sample and the set of proteins in ribosomes released from the membranes by treatment with puromycin-KCl (cf. Figs. 2b and 2c). Two other proteins (mol wt, 210,000 and 43,000, indicated by dots in Fig. 2) of nonribosomal origin also remained sedimentable after treatment of RM with 1% DOC. These proteins, however, were equally present in RM and SM samples. The other two polypeptides characteristic of RM (mol wt, 63,000 and 65,000) were solubilized by the DOC treatment and were found in the supernatant fraction (arrows in Fig. 2d), as expected for components of the microsomal membranes.

Several observations indicated that the 34,000 mol wt polypeptide represented urate oxidase, an enzyme which is the main component of peroxisomal cores (23, 39, 53). Electron microscope examination showed the presence of peroxisomal cores contaminating the RM fractions (Fig. 3a, arrowheads), but cores were absent from SM

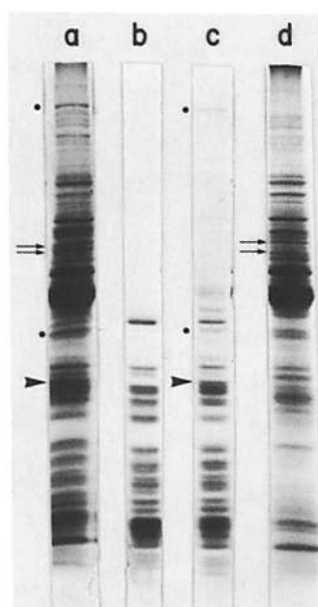


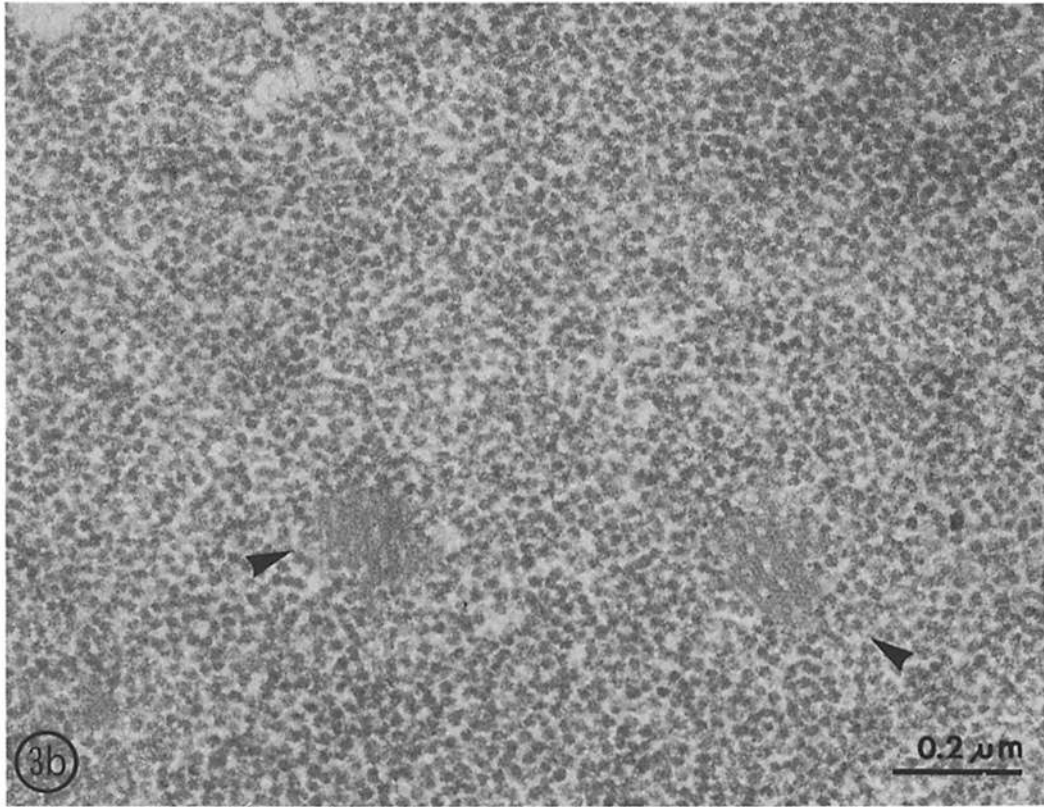
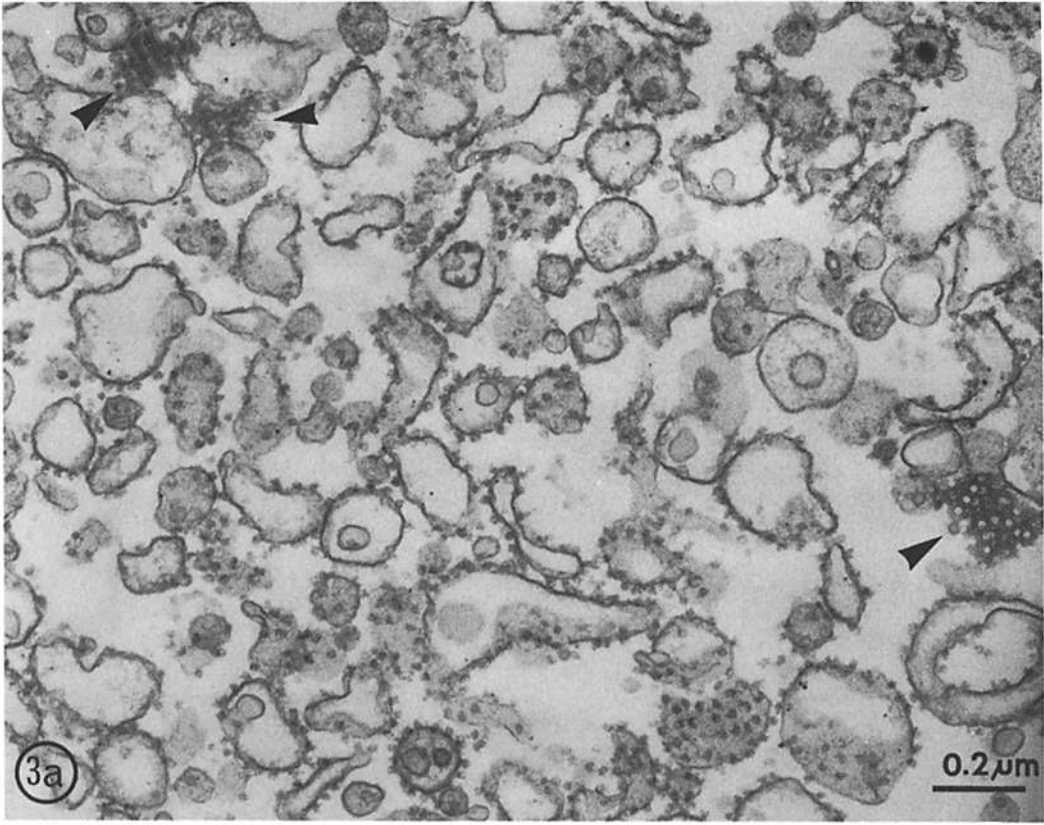
FIGURE 2 Distribution of microsomal proteins in subfractions obtained by puromycin-KCl or DOC treatment. (a) Total RM. (b) Ribosomes released by puromycin-KCl, recovered by sedimentation through a 2-ml cushion of 2 M SLSB (16 h-40K-Ti60). (c) Ribosomes obtained from RM solubilized with 1% DOC, recovered by sedimentation (60 min-40K-Ti50) through a 1 ml cushion of 20% SLSB. (d) Supernate obtained from DOC (1%)-treated RM after sedimentation of the ribosomes. Amounts derived from approx. 300 μ g of RM protein were loaded on SDS-acrylamide gradient gels (8-12%). In this and following figures, the position of the two proteins characteristic of RM designated as ribophorins are indicated by arrows. Arrowheads mark the position of urate oxidase, and dots indicate proteins with the mobility of myosin (upper dot) and actin (lower dot).

fractions (not shown). Peroxisomal cores are not easily solubilized by mild detergents (93, 98) and are therefore recovered with the ribosomes in the sedimentable fraction obtained from RM after DOC treatment (Fig. 3*b*, arrowheads). A comparison of SDS-acrylamide gel electrophoresis patterns showed that the protein of mol wt 34,000 which contaminates the RM (Fig. 5*a*) had the same mobility as urate oxidase in intact peroxisomes (arrowhead in Fig. 5*b*) or in cores prepared from purified peroxisomes (gift of Dr. B. Poole, The Rockefeller University, New York, New York) treated with DOC (arrowhead in Fig. 4). While rather pure preparations of peroxisomal cores containing urate oxidase could be obtained

by differential centrifugation of ribosome-stripped RM treated with DOC (Fig. 5*d*), a negligible amount of material containing no urate oxidase was recovered in sediments obtained from SM treated in a similar manner (Fig. 5*e*). Current work in our laboratory with Mr. E. Nack has provided definitive enzymological and immunological evidence identifying the 34,000 mol wt protein present in RM as contaminating peroxisomal urate oxidase.

As was mentioned above, two polypeptides of mol wt 210,000 and 43,000 (indicated by dots in Figs. 2 and 5) which are not ribosomal components, and are present in rough and smooth microsomal fractions, were recovered together with urate oxidase and the ribosomes in the sedimentable subfractions obtained from RM treated with 1% DOC. In their electrophoretic mobility, these polypeptides were identical to myosin (210,000 mol wt) and actin (43,000 mol wt), prepared from skeletal muscle, with which they were coelectrophoresed (not shown). It can not yet be established whether these proteins are contaminants of the microsomal fractions, or whether they are functionally related to the ER membranes. It should be noted, however, that several cytoskeleton proteins have been found in association with other cellular membranes (52, 68, 73, 74, 92).

The type of association which the proteins of mol wt 63,000 and 65,000 have with the microsomal membranes was examined applying current criteria to distinguish between peripheral and integral membrane components (84, 89). It was found (Fig. 6) that the association of these polypeptides with the membranes was not affected by treatment of the microsomes with alkaline (0.025 N or 0.1 N NaOH) or acidic solutions (0.5 N acetic acid) or with EDTA (not shown). Removal of proteins by these procedures has been proposed as a criterion to establish the peripheral character of a membrane protein (89). As can be seen in Fig. 6, a comparison of gel patterns of supernate(s) from microsomes treated with NaOH or acetic acid (HAC) with patterns of content proteins released from microsomes by low concentrations of Triton X-100 (1.3×10^{-3} M), shows that the acidic, and especially the alkaline treatments, very effectively removed content proteins from the microsomes. In all cases, however, the two polypeptides characteristic of RM (mol wt, 63,000 and 65,000, marked by arrows) remained associated with the membranes. Although the



effectiveness of the alkaline treatment in removing content and peripheral proteins increased with the NaOH concentration, similar sets of proteins were released in 0.1 N and in 0.025 N NaOH (Fig. 6). In addition to the content proteins, the set of proteins released by alkali (and to a lesser extent, that released by acetic acid) included the contaminating urate oxidase (arrowhead in Fig. 6), as expected from observations with peroxisomal fractions (53, 57, 93).

Isolation of Microsomal Membrane Polypeptides Associated with Bound Ribosomes

Observations described in the preceding section indicated that RM contain two polypeptides which are not present in SM and are integral components of the microsomal membranes. We investigated the possibility that these polypeptides are related to the presence of binding sites for ribosomes in membranes derived from the rough endoplasmic reticulum. The ionic nature of the direct bonds between ribosomes and membrane components (4) suggested to us the possibility that, by using nonionic detergents to dissolve the membranes of RM, it might be possible to isolate ribosomes still associated with protein components of the binding sites.

A survey of detergents indicated that several nonionic detergents (Brij 35, Triton X-100, and Kyro EOB) produced an incomplete solubilization of microsomal proteins. This was the case even when the detergents were applied at concentrations (2.5×10^{-2} M) which led to an almost complete solubilization of microsomal phospholipids labeled *in vivo* with [3 H]choline. SDS-acrylamide gel analysis of the undissolved subfractions obtained by sedimentation showed that, after treatment with 2.5×10^{-2} M Kyro EOB, the polypeptides of mol wt 63,000 and 65,000 were recovered quantitatively with the sedimentable ribosomes (Fig. 7*d*). Other detergents either caused partial solubilization of these polypeptides (Triton X-100) or resulted in poor solubilization

of a more extensive set of microsomal proteins (Brij 35).

A comparison of protein bands in gels from supernates and sediments obtained by differential centrifugation from RM treated with 2.5×10^{-2} M Kyro EOB (Fig. 7*b* and *d*) or DOC (Fig. 7*a* and *c*) showed that the presence of the two bands (marked by arrows in gels of Kyro EOB residues (Fig. 7*d*) and DOC supernates (Fig. 7*a*)) constituted the main difference in the composition of the subfractions. It was also clear that Kyro EOB did not lead to the solubilization of any protein not solubilized by DOC. Thus, in addition to the ribosomal proteins, urate oxidase (arrowhead in Fig. 7*d*), and polypeptides with the mobility of actin and myosin (marked by dots in Fig. 7*d*) were present in sediments obtained with both detergents (Figs. 7*c* and *d*). A comparison of gels of the solubilized subfractions (Fig. 7*a* and *b*) shows that, in addition to the polypeptides characteristic of the membranes of RM (Fig. 7*a*, arrows), a microsomal protein of small mol wt ($\sim 15,000$, indicated by an asterisk in Fig. 7*a* and *d*) was solubilized by DOC but not by the Kyro EOB treatment. This polypeptide, however, was present in both SM and RM (cf. Fig. 1*a* and *d*). Quantitatively minor differences affecting the intensity of other bands present in supernates obtained by both detergents may also be noted in Fig. 7. These may indicate differential effects of both detergents on the solubilization of the corresponding polypeptides.

Chemical determinations were carried out in aliquots of the same sedimentable fractions used for the electrophoretic analysis shown in Fig. 7*c* and *d*. A comparison of the results with both residues shows (Table I) that, as expected, almost all the microsomal RNA was present in the sediments containing the ribosomes. The more effective solubilization of the microsomal membranes caused by DOC was apparent in the different protein and phospholipid recoveries. The RNA/protein ratio (0.95) in residues obtained after DOC treatment was similar to that of crude ribosome preparations. The RNA/protein ratio

FIGURE 3 Peroxisomal cores contaminating RM (*a*) and bound ribosome samples (*b*). RM (*a*) were fixed in suspension with 1% glutaraldehyde sedimented (20 min-10K-SW56) and postfixed in 1% OsO₄. Polysomes (*b*) (1%) were sedimented (2 h-40K-Ti50), and fixed as a pellet. Micrographs were taken from the lower portions of the pellets which have a higher concentration of peroxisome cores (arrowheads). (*a*) $\times 60,000$; and (*b*) $\times 85,000$.

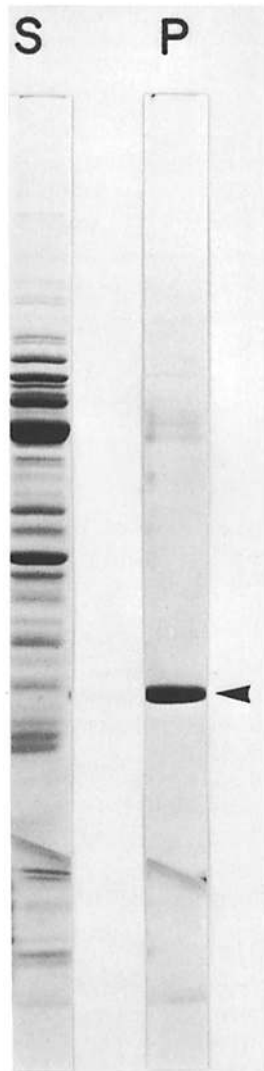


FIGURE 4 Urate oxidase in peroxisomal cores prepared from purified peroxisomes. A sample of purified peroxisomes was resuspended in LSB (2 mg/ml), treated with DOC (2.5×10^{-2} M), and incubated for 30 min at 4°C . Cores were recovered by sedimentation (60 min-40K-Ti50) through a 0.3-ml cushion of 20% SLSB. The pellet (*P*), resuspended in 1.3 ml of water, and the supernate (*S*) were prepared for SDS-acrylamide gel electrophoresis, and aliquots derived from approx. 200 μg of peroxisomal protein were loaded onto SDS-acrylamide gradient gels (8-12%). The band corresponding to urate oxidase in the pellet fraction (*P*) is marked by an arrowhead.

(0.68) in Kyro EOB residues reflects not only the retention of the proteins of mol wt 65,000 and 63,000 but also a generally less complete solubili-

zation of the microsomal proteins. This was also apparent in the higher content of microsomal cytochromes and NADPH cytochrome *c* reductase in Kyro EOB residues (from 5 to 16% of the initial values).

The demonstration that polypeptides characteristic of RM are present in sedimentable fractions obtained by differential centrifugation from Kyro EOB treated RM is not sufficient to establish unequivocally their association with the ribosomes. Therefore, RM treated with Kyro EOB or DOC were fractionated by sedimentation in sucrose density gradients to rule out the possibility that microsomal proteins not solubilized by the detergent sedimented independently of the ribosomes. RM which were labeled in vivo with [^3H]choline (4 h) were used to assess, simultaneously with the sedimentation patterns, the release of phospholipids which accompanies the dissolution of the membranes (Fig. 8). A striking difference in the sedimentation profiles of samples

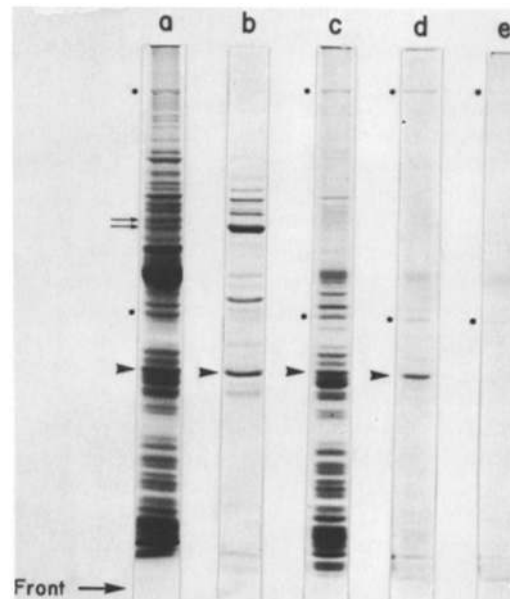


FIGURE 5 Identification of urate oxidase in peroxisomal cores contaminating RM. Freshly prepared RM, RMstr, and SM were resuspended in LSB (3.5, 3, and 3 mg protein/ml, respectively) and treated with DOC (2.5×10^{-2} M). Residues recovered by sedimentation (40 min-40K-Ti50) through a 1-ml cushion of 20% SLSB were resuspended in water. Samples derived from 200 μg of total microsomal protein were loaded onto SDS-acrylamide gels (7-12%). (*a*) Total RM; (*b*) 100 μg of purified peroxisomes; and (*c-e*) residues from RM (*c*), RM stripped (*d*), and SM (*e*) treated with DOC.

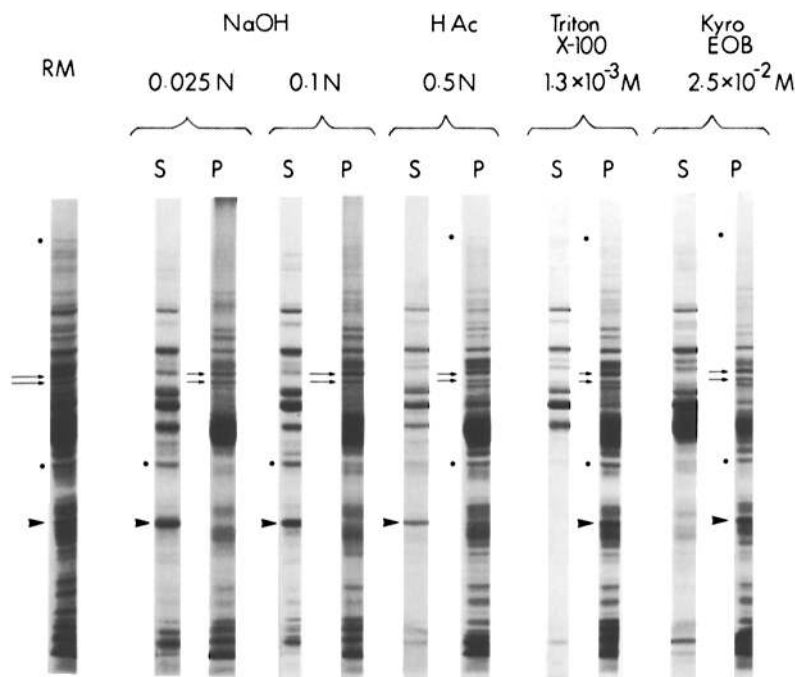


FIGURE 6 Identification of integral membrane proteins in RM. RMstr resuspended in water (3 mg protein/ml) were treated with NaOH, acetic acid, or Triton X-100 at the concentrations indicated. Pellets obtained after differential centrifugation (90 min-35K-Ti50) were resuspended in the original volume of water. Supernate (*S*) and pellet (*P*) fractions derived from 300 μ g of RM were analyzed in SDS-acrylamide gradient gels (7-12%).

treated with DOC or Kyro EOB became apparent (Fig. 8). As is usually the case when no RNase inhibitors are added before detergent treatment, monomeric ribosomes and poorly preserved polysomes predominated in samples of bound polysomes prepared by DOC (Fig. 8a). On the other hand, large ribosomal aggregates resembling very well preserved polysomes were present after treatment of RM with Kyro EOB (Fig. 8b).

Electrophoretic analysis of fractions taken from gradients (Fig. 9) clearly showed that the polypeptides characteristic of RM behaved as membrane proteins which, after phospholipids and other proteins are dissolved, remain directly associated with the ribosomes. The two polypeptides were associated with monomeric ribosomes and ribosomal aggregates found within the gradients (Fig. 9b and c) and with large aggregates which rapidly sedimented to the bottom of the tubes (Fig. 9d). They were absent, however, from gradient fractions near the meniscus which contained solubilized proteins (Fig. 9a). This distribution indicates that the two proteins do not simply cosediment with the ribosomes, as would be expected of

membrane components insoluble in Kyro EOB, or of aggregates or microsomal contaminants. Other microsomal polypeptides which were present in Kyro EOB residues obtained by differential centrifugation (Fig. 7d) were, on the other hand, not found with the monomeric ribosomes or with the pseudopolysomal aggregates within the gradients. These polypeptides were present exclusively at the bottom of the tubes together with the fast sedimenting residue. Some represent incompletely dissolved membrane proteins unrelated to the ribosomes, such as cytochrome P-450 (intense bands present in all samples near the middle of the gels), while others are nonmicrosomal protein, such as urate oxidase (arrowhead in Fig. 9d), actin or myosin (dots in Fig. 9d).

To determine whether there was a relationship between the amount of the two polypeptides and the number of ribosomes in the aggregates, fractions taken from different regions of the gradients were pooled and aliquots of equal ribosome content were analyzed by SDS-acrylamide gel electrophoresis. Densitometric tracings (not shown) of the gels (Fig. 9b-d) showed that,

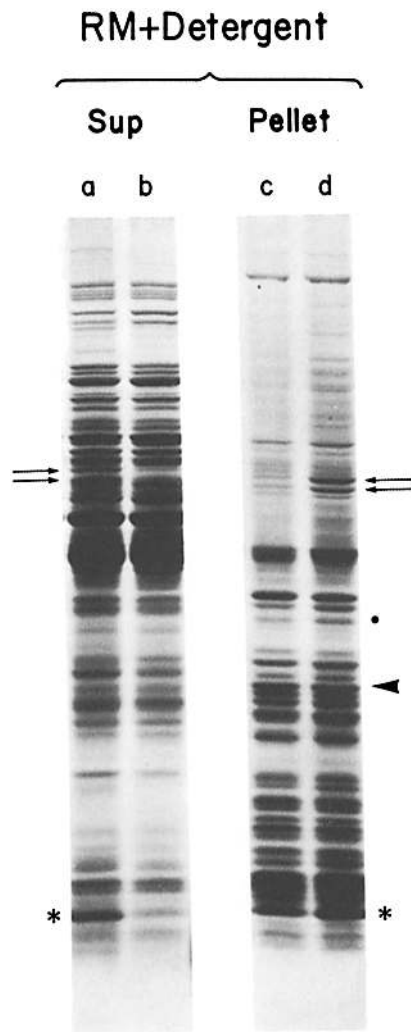


FIGURE 7 Differential effect of DOC and Kyro EOB on the solubilization of microsomal proteins. RM resuspended in LSB (3.5 mg protein/ml) were incubated (30 min, 4°C) with 2.5×10^{-2} M DOC (a and c) or Kyro EOB (b and d) and fractionated by sedimentation (60 min-40K-Ti50). Aliquots of the supernate (a and b) and pellet fractions (c and d) derived from 350 μ g of RM protein were analyzed in SDS-acrylamide gradient gels (8-12%). The two proteins characteristic of RM are found in the DOC supernate (arrows in gel a) but are recovered with the sedimentable ribosomes after Kyro EOB treatment (arrows in gel d). The contaminating protein urate oxidase (arrowhead) and the proteins comigrating with actin and myosin (dots in d) are found in both sedimentable fractions. The protein represented by a band marked with an asterisk is present in Kyro EOB residues (a) and DOC supernates (d).

TABLE I
Composition of RM and the Sedimentable Subfractions Obtained after Treatment with Detergents*

	Control	DOC	Kyro EOB
Protein	100% (2.98 mg)	25%	36%
Phospholipid (PL)	100% (1.13 mg)	0.05%	9%
PL	0.38	0.06	0.09
Protein	100% (0.75 mg)	94%	97%
RNA	0.251	0.946	0.679
Protein	100% (2.81 pmol/mg protein)	4%	16%
Cytochrome P-450, 420	100% (1.12 pmol/mg protein)	2%	10%
Cytochrome b_5	100% (0.20/U/mg protein)	3%	5%
NADPH-Cytochrome c -reductase			

* RM resuspended in LSB (3.4 mg protein/ml) were incubated for 30 min at 4°C with water (control) 1% DOC (2.5×10^{-2} M) or 1.5% Kyro EOB (2.5×10^{-2} M). Samples were centrifuged (60 min-30K-#40), and pellets were analyzed.

because of the similar ribosomal content, bands representing ribosomal proteins (in the lower half of the gels) had similar intensities in all three samples. This was also the case for the two proteins characteristic of the RM membranes (marked by arrows in Figs. 9b-d). The intensity of these bands was compared with that of a ribosomal protein taken as a reference (marked with an asterisk in Fig. 9d) in order to calculate an approximate stoichiometric relationship. Taking into account the molecular weight differences and assuming that the staining intensity of bands in gels is a measure of the amount of protein, and that ribosomal and microsomal membrane proteins have similar specific staining intensities, it was estimated that approx. 1.4-1.9 copies of each of the two microsomal proteins of mol wt 63,000 and 65,000 was present in the RM membranes per bound ribosome. Although this estimate must be considered tentative, a similar relationship between ribophorin and ribosome content was calculated when the absorbance due to total ribo-

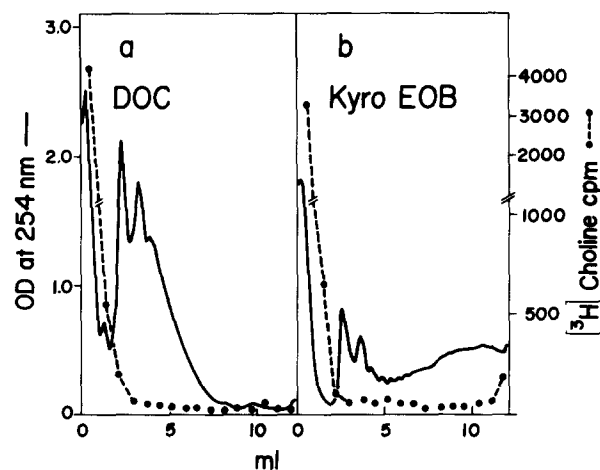


FIGURE 8 State of ribosome aggregation in RM treated with DOC or Kyro EOB. RM containing phospholipids labeled in vivo (^3H choline, 4 h) were resuspended in LSB (3 mg protein/ml). Aliquots received 2.5×10^{-2} M DOC (a) or Kyro EOB (b). Samples were layered onto linear sucrose gradients (10–60% SLSB). After centrifugation (90 min-40K-SW41), absorbance (254 nm) profiles were recorded and fractions collected throughout the gradients. ^3H -radioactivity was determined in 100- μl aliquots.

somal proteins measured in gels of isolated bound polysomes was compared with absorbance of ribophorins measured in gels of equivalent amounts of RM or in gels of Kyro EOB residues obtained from them.

These observations suggest a close relationship between the two proteins characteristic of RM and the presence of ribosome-binding sites in rough endoplasmic reticulum membranes. It will be shown in the following paper (46) that in intact microsomes the proteins characteristic of rough membranes can be cross-linked to the ribosomes by bifunctional reagents. These proteins are therefore present at, or near, the ribosome binding sites in the native membranes. For these reasons, they were named ribophorins I and II (ribophorin I: 65,000 mol wt; ribophorin II: 63,000 mol wt) and for convenience these names will be used in the rest of this paper.

State of Aggregation of Ribosomes after Extraction of Membrane Proteins

To investigate the relationship between the two ribophorins and the ribosomes in Kyro EOB sediments, several treatments which are known to normally produce polysome breakdown, disassembly of ribosomes, and/or removal of ribosomes from microsomal membranes were applied. Incubation of Kyro EOB residues with pancreatic RNase in a medium of low salt concentration did

not affect the state of aggregation of the ribosomes (Fig. 10c). This insensitivity to RNase established that the ribosomal aggregates do not simply represent well preserved polysomes. It was also demonstrated independently, using free polysomes (not shown), that: (a) Kyro EOB is not an inhibitor of RNase and (b) this detergent does not produce an artefactual aggregation of ribosomes. These findings indicate that the integrity of mRNA is not necessary for the maintenance of the large ribosomal aggregates.

Several observations indicated that the pseudo-polysomal patterns observed after Kyro EOB treatment represented ribosomal aggregates maintained by interactions which are sensitive to high salt and anionic detergents and are mediated by proteins. When Kyro EOB residues were treated with DOC (Fig. 10b) or incubated in a medium of high ionic strength (Fig. 10d), sedimentation patterns corresponding to poorly preserved polysomes containing mainly monosomes, dimers, and small-size aggregates were obtained (Fig. 10b and d). Kyro EOB residues were very sensitive to limited proteolytic digestion with trypsin (10 $\mu\text{g}/\text{ml}$ at 4°C for 30 min) which led to the release of ribosomal particles sedimenting somewhat heterogeneously in the monomer and dimer region (Fig. 10f). SDS-gel electrophoretic analysis of Kyro EOB residues incubated with trypsin demonstrated that both ribophorins were accessible to

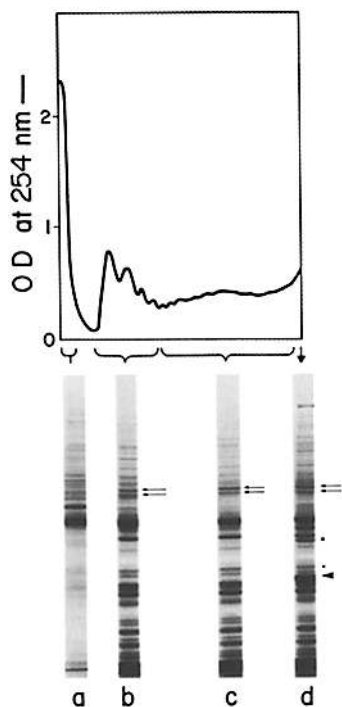


FIGURE 9 Association of ribosomes in a constant stoichiometric ratio with the two ribophorins. RM were resuspended in LSB (3.5 mg/ml) and incubated for 30 min at 4°C with Kyro EOB (2.5×10^{-2} M). A 0.5-ml sample was analyzed by sucrose density gradient centrifugation (60 min-40K-SW41) in a linear 10-60% SLSB gradient containing Kyro EOB (1.25×10^{-2} M). The absorbance profile (254 nm) was monitored and 1-milliliter fractions were collected. Fractions from different regions of the gradient (*a*, *b*, and *c*, indicated by brackets) were pooled. Fractions *b* and *c* were diluted with LSB, and the sedimentable material was recovered (14 h-40K-Ti60). Samples containing equivalent amounts of ribosomes (OD_{260} U) from fractions *b*, *c*, and the resuspended pellet were analyzed in SDS-acrylamide gels (8-12%). The position of the ribophorins in fractions *b*, *c*, and *d* is indicated by arrows. Contaminants found at the bottom of the gradient are marked by an arrowhead (urate oxidase) and the proteins with the electrophoretic mobility of myosin and actin (dots) are found at the bottom of tube (*d*). The Coomassie blue-staining band corresponding to a ribosomal protein indicated by a star in gel *d* was used to estimate the number of ribophorins per ribosome.

the enzyme and very sensitive to the proteolytic attack. Even after very brief periods of incubation with the protease, bands corresponding to the ribophorins disappeared almost completely from their normal positions in the gels (Fig. 11). As is shown in Fig. 11, limited trypsinization also

caused changes in the mobility of several other membrane and ribosomal polypeptides.

Treatment of the Kyro EOB residues with puromycin-KCl resulted in the complete dissociation of the ribosomes into ribosomal subunits (Fig. 10*e* and Fig. 12*a*) and, as expected from the behavior of ribosomes bound to microsomal mem-

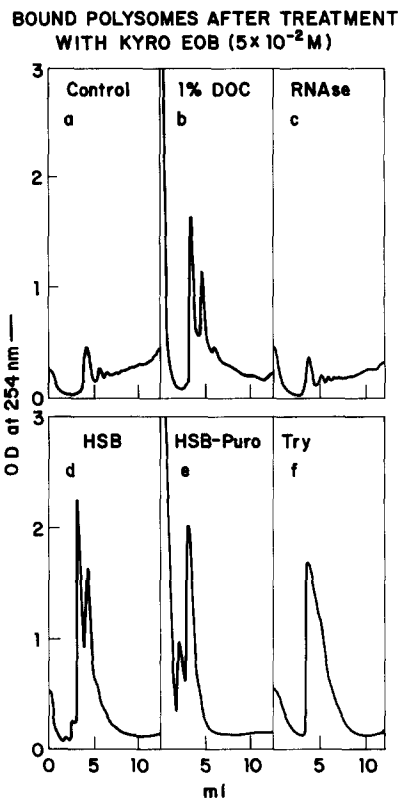


FIGURE 10 Effect of various treatments on the state of ribosome aggregation in Kyro EOB residues. RM were resuspended in LSB treated with Kyro EOB (2.5×10^{-2} M) and sedimented (60 min-40K-Ti60). After resuspension in one-half the original volume of 10 mM Tris-HCl pH 7.6, 225- μ l aliquots received: (a) 225 μ l $2 \times$ LSB + 50 μ l LSB (30 min, 4°C); (b) 225 μ l $2 \times$ LSB + 50 μ l 10% DOC (30 min, 4°C); (c) 225 μ l $2 \times$ LSB + 50 μ l RNase (5 μ g/ml) (30 min, 4°C); (d) 225 μ l $2 \times$ HSB + 50 μ l HSB (30 min, 4°C); (e) 225 μ l $2 \times$ HSB + 50 μ l Puromycin (10^{-2} M) (15 min, 30°C); and (f) 225 μ l $2 \times$ LSB + 50 μ l Trypsin (30 min, 4°C) (100 μ g/ml).

Samples *a*, *b*, *c*, and *f* were loaded onto linear sucrose gradients containing LSB (10-60%) SLSB while samples *d* and *e* were analyzed on gradients containing high salt buffer (10-50% SHSB). After centrifugation (90 min-40K-SW41), absorbance (245 nm) profiles were recorded.

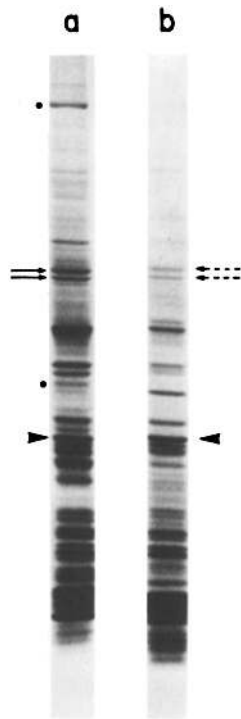


FIGURE 11 Sensitivity of ribophorins in the Kyro EOB residue to trypsin digestion. RM resuspended in LSB were treated with Kyro EOB (2.5×10^{-2} M). Half of the sample was left as a control (a) while the other half received trypsin (10 $\mu\text{g}/\text{ml}$) (b). After incubation (60 min, 0°C) (50 $\mu\text{g}/\text{ml}$) TLCK (a trypsin inhibitor) was added to both samples. Sedimentable fractions were prepared (60 min-40K-Ti50), and aliquots of the resuspended sediments were analyzed in SDS-acrylamide gradient gels (8-12%).

branes (4) in the release of the ribophorins (see below and Fig. 12 b). Electrophoresis of fractions collected from sucrose density gradients (Fig. 12 b) showed that the ribophorins remained aggregated and sedimented rapidly to the bottom of the tubes together with the detergent-insoluble proteins present in the microsomal samples (urate oxidase, actin, and myosin), while the ribosomal subunits were free of microsomal proteins. For comparison, it should be noted that when DOC residues are treated with puromycin-KCl the set of proteins which remains rapidly sedimentable does not include the ribophorins (Fig. 11 a).

It was also possible to isolate ribophorin complexes bearing no ribosomes by detergent treatment of microsomal membranes which had been previously stripped of ribosomes by the puromycin-KCl procedure (RMstr). It has previously

been shown (38, 94) that cholate is a milder detergent than DOC, which can be used to extract native protein complexes from liver plasma membranes. Indeed, cholate was more effective than Kyro EOB in producing a more complete solubilization of membrane proteins, other than the ribophorins from RMstr. As is shown in Fig. 13 most proteins solubilized by cholate were found in the top fractions of the gradients (fractions 1 and 2). On the other hand, the ribophorins sedimented together as complexes of heterogeneous

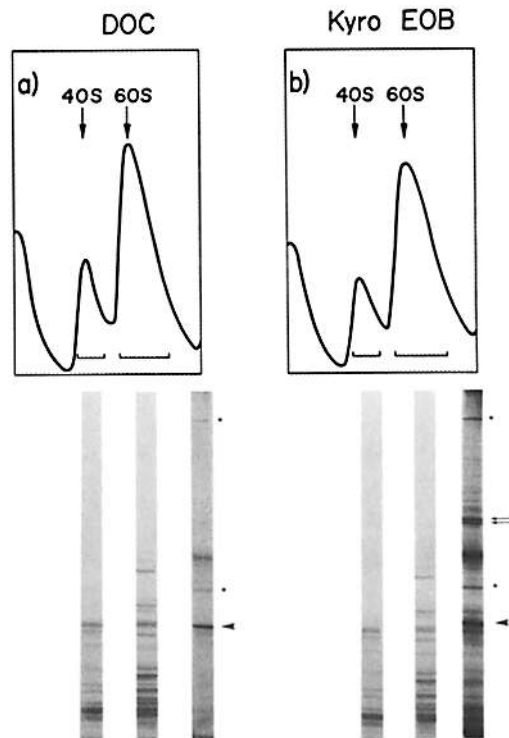


FIGURE 12 Release of ribophorins from Kyro EOB sediments upon dissociation of ribosomes into subunits. Aliquots of RM resuspended in LSB (3 mg/ml) were treated with 2.5×10^{-2} M DOC (a) or Kyro EOB (b). After incubation for 30 min at 4°C , samples (1 ml) were underlayered with 0.5 ml of 20% SLSB and centrifuged (90 min-40K-Ti50). Sediments were resuspended in HSB, 2.5, and incubated with puromycin (10^{-3} M, 20 min, 30°C). 0.5-ml aliquots were used for sedimentation in a 10-25% SHSB gradient (165 min-35K-SW41- 22°C). Absorbance at 254 nm was recorded, and fractions corresponding to the 40S and 60S ribosomal subunit peaks were collected, pooled, and sedimented (16 h-40K-Ti60). Aliquots of all samples including the pellets from the sucrose density gradient centrifugation were prepared for SDS-acrylamide gel electrophoresis (8-12%).

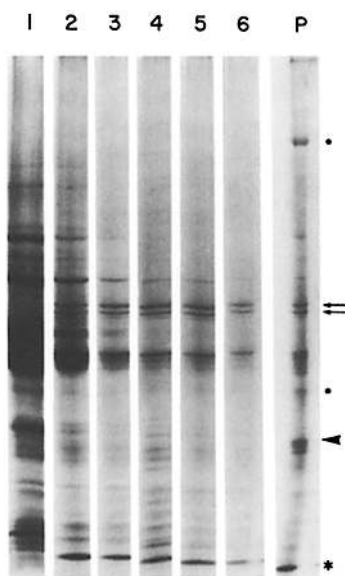


FIGURE 13 Isolation of ribophorins in sedimentable complexes obtained from RM previously stripped of ribosomes (RMstr). A sample of RMstr (3 mg protein/ml) in 25 mM phosphate (pH 7.25) containing 10 mM EDTA was treated with 1% Na-cholate and incubated for 30 min at 0°C. Aliquots (1 ml) were layered onto linear sucrose density gradients (10–30% sucrose, 25 mM phosphate, 10 mM EDTA, 0.5% cholate). After centrifugation (3 h-25K-SW27), fractions were collected and incubated with 5% TCA at 4°C. TCA precipitates (fractions 1–6) and the pellet (P) of the sucrose density gradient were dissolved in electrophoresis sample buffer, and aliquots were analyzed on SDS-acrylamide gels (8–13%). A protein of low molecular weight (asterisk in gel P) was to a variable extent always associated with the sedimentable complexes containing ribophorins.

size found throughout the gradients (fractions 2–6 and P in Fig. 13). These complexes also contained other microsomal membrane proteins, mainly a protein of mol wt 50,000 (with a mobility similar to cytochrome P-450) and a protein of mol wt 15,000 (indicated by an asterisk in Fig. 13, column P). By this procedure, urate oxidase (arrowhead, Fig. 13, column P), myosin and actin (dots,

Fig. 13, column P) were also recovered at the bottom of the tubes.

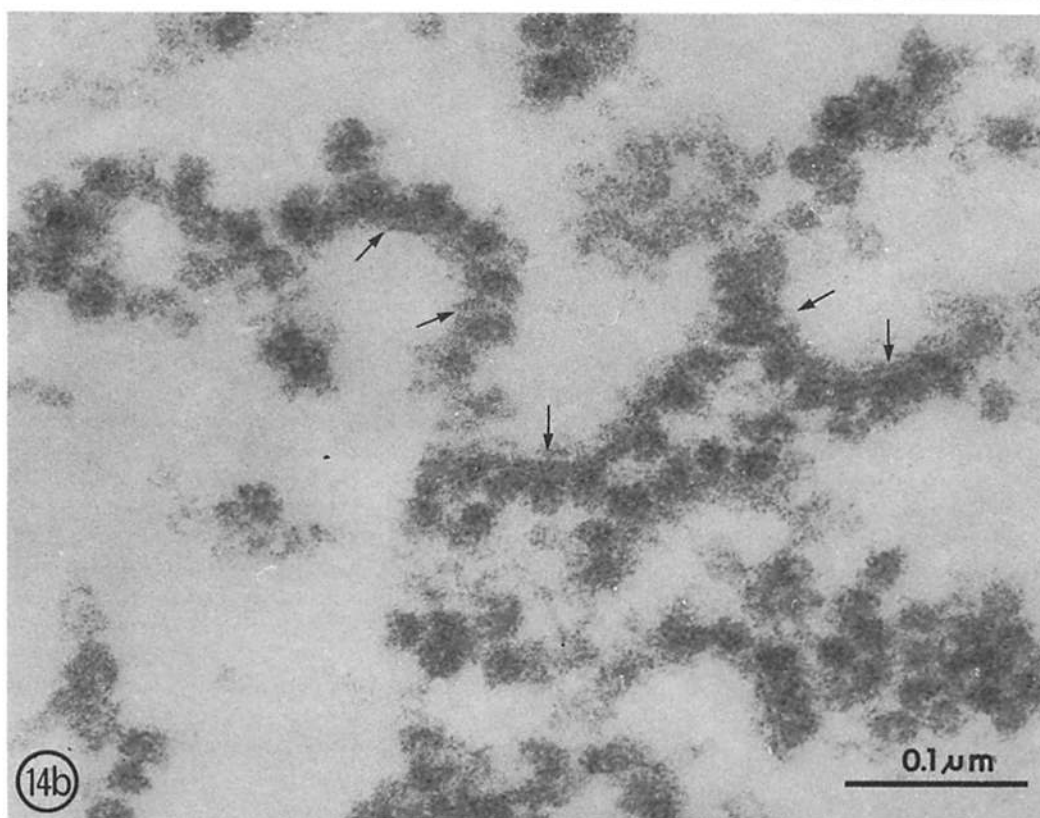
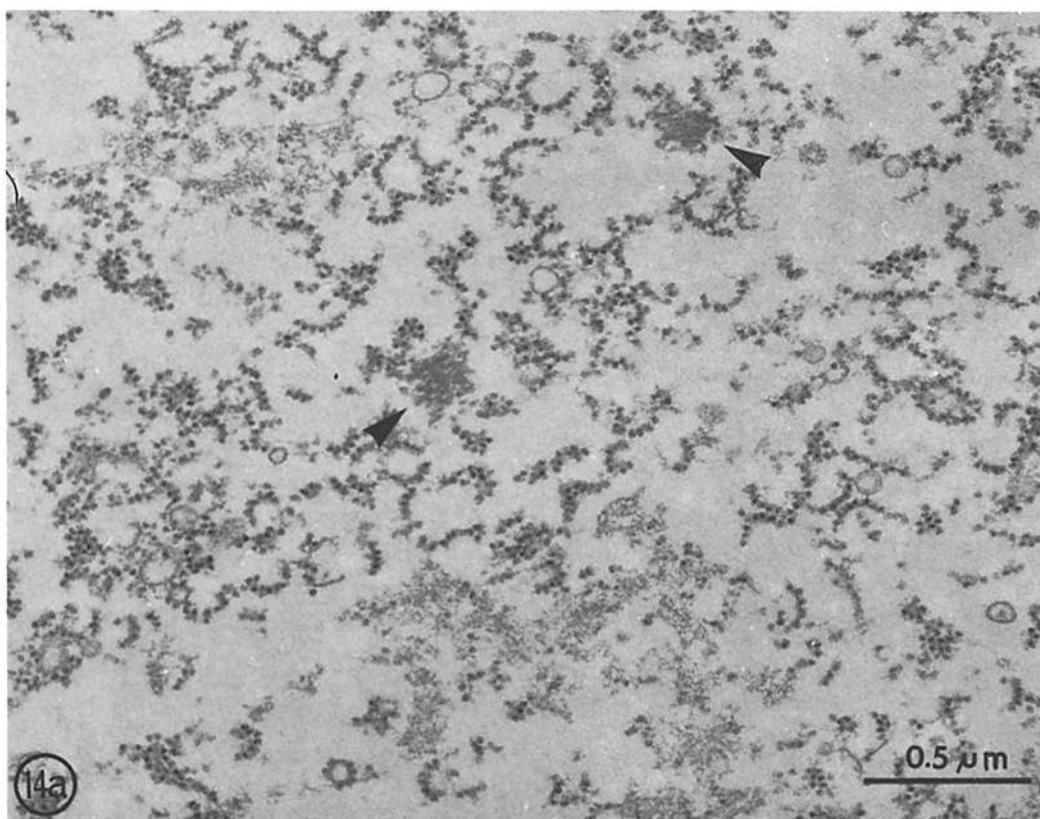
Electron Microscopy

Electron microscope examination of sediments obtained from RM treated with 2.5×10^{-2} M Kyro EOB showed that the pseudopolysomal aggregated consisted of groups of tightly packed ribosomes attached to the convex side of curved membrane remnants (Figs. 14a and b). The number of ribosomes seen on the curved profiles varied from a few to more than 30. Membrane remnants under the ribosomes appeared as layers of amorphous material or presented a trilaminar unit membrane appearance, which was reminiscent of the original ER membrane. The thickness of the layer (100 Å), however, was considerably greater than that of normal microsomal membranes (55–70 Å). Usually, membrane remnants bearing ribosomes were cup-shaped. Ribosome patches were visible when remnants were sectioned tangentially, and images of closed circles bearing ribosomes on the outside were frequent. Occasionally, in between the ribosomal aggregates, membrane remnants not bearing ribosomes but with a trilaminar appearance were also observed. These usually produced straight or only slightly curved profiles.

DISCUSSION

Rough and smooth microsomes are vesicles derived during homogenization from portions of the endoplasmic reticulum characterized, respectively, by the presence or absence of ribosomes bound to the membranes (71). Several studies have demonstrated that many membrane proteins and proteins of the vesicular lumen are similar in both microsomal fractions and that the major compositional difference between rough and smooth microsomes is due to the presence of ribosomal proteins in the former (7, 12, 21, 24, 37, 47–49, 61, 62, 86, 96, 97, 100). Although qualitative and quantitative compositional differences between

FIGURE 14 Thin-section electron micrographs of the sedimentable subfraction obtained from RM treated with 2.5×10^{-2} M Kyro EOB in a low salt buffer. The resuspended sediment was fixed in suspension with 1% glutaraldehyde collected by filtration on a millipore filter (8) and postfixed with 1% OsO₄. Groups of tightly packed ribosomes remain attached to remnants of the microsomal membranes. (a) Arrowheads point to cores of peroxisomes contaminating the preparation. $\times 45,000$. (b) Arrows point to the remnants of the endoplasmic reticulum membrane to which ribosomes remain bound. $\times 240,000$.



membranes of rough and smooth microsomes have been reported (7, 43, 83), it has not been excluded that these reflect the presence of contaminants in RM derived from other organelles such as mitochondria, peroxisomes, Golgi apparatus, and plasma membranes.

Previous studies have shown (18) that the capacity of rough microsomal membranes stripped of ribosomes by treatment with puromycin-KCl to rebind ribosomes *in vitro* is sensitive to proteolysis and to heat denaturation, but is unaffected by incubation of the microsomes with phospholipase or treatments which remove peripheral membrane proteins. For these reasons, it was suggested (18) that integral membrane proteins specific to the rough ER are important components of the binding sites in these membranes. We have shown that membranes of RM contain two integral polypeptides (mol wt, 63,000 and 65,000) which are not found in SM. In addition to their subcellular distribution, several observations suggest that these polypeptides are related to the sites for ribosome binding. Although the polypeptides were solubilized by DOC, both were recovered together with the ribosomes when neutral detergents were used to dissolve the microsomal membranes. The detergent Kyro EOB, a neutral polyethoxyalkylether, which was first used to subfractionate free and membrane-bound polysomes in tissue culture cells (11), allowed the extraction of most other proteins and phospholipids from the membranes without disrupting the association of the two polypeptides with the ribosomes. In the residues obtained after Kyro EOB treatment, the two polypeptides were present in a fixed stoichiometric ratio with respect to the number of ribosomes, and were part of membrane remnants to which the ribosomes were directly attached. Because these properties of the two polypeptides characteristic of RM suggested that they participate in ribosome binding, they were designated ribophorins (I and II).

The most direct interpretation of the results just summarized is that the interaction of the ribophorins with the ribosomes precedes the solubilization of the membrane phospholipids and the extraction of other membrane proteins caused by Kyro EOB and other neutral detergents. It cannot be excluded from the evidence presented in this paper, however, that the ribophorins become associated with the ribosomes only when other membrane components are removed by the detergent. In fact, it is apparent from the close packing of

ribosomes in the membrane remnants obtained after Kyro EOB treatment that the distribution of the binding sites in the plane of the rough membranes is greatly altered by the extraction procedure.

Results in the following paper (46) show, however, that the association of the ribophorins with the ribosomes is not a fortuitous one, which may be established only after the binding sites are reorganized; in intact RM, ribophorins could be cross-linked to the membrane-bound ribosomes by short bifunctional reagents, as expected of membrane components closely associated with the binding sites. The results in this and the following paper (46), therefore, suggest that the ribophorins either directly provide binding sites for the ribosomes or contribute, in association with other membrane components, to the integration of supramolecular assemblies which act as ribosome receptors.

In several respects, the association of the ribosomes with the ribophorins within the aggregates obtained after Kyro EOB treatment resembles that of ribosomes with sites in the original membranes. Thus, ribosomes and polysomes within the aggregates were released in media of high ionic strength, when Mg^{++} ions were chelated by EDTA and by treatment with the anionic detergent DOC, all conditions which break the direct ionic bonds between large ribosomal subunits and the binding sites in the membranes (4). On the other hand, puromycin, which is necessary to detach active ribosomes from native microsomal membranes, was not necessary to break the association of ribosomes with the ribophorins within the Kyro EOB aggregates. This indicates that the link between ribosomes and membranes, which in the native microsomes is provided by the nascent chains, is no longer present in the aggregates. On this basis, it may be suggested that components which are removed by the detergent, possibly membrane phospholipids, normally interact with the intramembranous portions of nascent polypeptides. Alternatively, it is also possible that a direct interaction between the ribophorins and the nascent polypeptides which exists in the native microsomes is disrupted when the architecture of the membranes is altered by the Kyro EOB treatment.

Other investigators have reported compositional differences between rough and smooth microsomes which were interpreted as reflecting the presence of ribosome receptors in the rough mem-

branes. Bailey et al. (7) have emphasized the presence of a polypeptide of approx. 36,000 daltons in rat liver RM samples, which is absent or less prominent in SM. This polypeptide was interpreted as representing a membrane protein which may be of ribosomal origin or is involved in ribosome attachment (7). The electrophoretic mobility of the polypeptide observed by Bailey et al. (7) is similar to that of urate oxidase (34,000 daltons) which we identified as a contaminant of RM fractions present in the cores of peroxisomes (23, 53, 93, 98). Because, in cell fractionation media, rough microsomal vesicles have a tendency to form aggregates (3, 22) in which peroxisomal cores may be trapped, urate oxidase could not be easily eliminated as a contaminant of RM by sedimentation or even by flotation of the microsomes through a heavy sucrose cushion. Treatment with alkaline solutions (53, 57, 93), however, resulted in the solubilization of the peroxisome cores and in the release of the urate oxidase together with the content of the microsomal vesicles, without causing a significant removal of the ribophorins or other integral proteins from the membranes. Since cores of peroxisomes are not easily solubilized by detergents (93, 98), urate oxidase was also found to be present in samples of bound ribosomes obtained by detergent treatment from RM. In these samples, the contaminant could be erroneously interpreted as representing a ribosomal protein characteristic of bound ribosomes or as a protein which remains associated with the ribosomes and is related to their binding to the membrane. The 34,000 mol wt polypeptide, however, is not found in samples of ribosomal subunits obtained from RM by treatment with puromycin-KCl, which have the same protein composition as subunits obtained by dissociation of free polysomes (54).

Two other polypeptides were present in the microsomal fractions which were consistently recovered with the ribosomes after detergent treatment of RM. These were represented in SDS gels by bands with the electrophoretic mobility of myosin and actin from skeletal muscle (mol wt, 210,000 and 43,000, respectively). It cannot yet be ascertained whether these cytoplasmic polypeptides, which were present in both rough and smooth microsome samples, were fortuitously associated with the microsomes, and therefore should be regarded as contaminants, or whether they are functionally related to the membranes as is the case with contractile and cytoskeleton pro-

teins in other systems (52, 68, 73, 74, 92).

Several attempts have recently been made to exploit the affinity of ribosomes for their binding sites to purify specific components of ER membranes involved in ribosome binding. Microsomal lipids and membrane proteins have been extracted from RM in the expectation that components of the binding sites may remain associated with the ribosomes (28, 34, 65). Fujita et al. (34) have observed that polysomes recovered from RM treated with Triton X-100 in a medium of low salt concentration carry extraneous (nonribosomal) proteins which may be removed by subsequent incubation with DOC. These authors reported that incubation of purified ribosomes with the material extracted by DOC from polysomes prepared with Triton X-100 resulted, upon pre-equilibration with Triton X-100, in the adsorption of several proteins (mol wt 108,000, 99,000, and 65,000) to the ribosomes which were presumed to be related to ribosome binding (34). Although at least ribophorin I may be one (mol wt, 65,000) of the proteins adsorbed to the ribosomes in these experiments, the significance of the adsorption of other proteins of higher molecular weight (108,000, 99,000) remains unexplained. Since these proteins do not appear to be specific components of the RM, it is possible that their association with the ribosomes is mediated by the ribophorin(s) and is therefore indirect. Cross-linking experiments or studies to determine whether a compulsory order of addition of the proteins is necessary for their association with the ribosomes have not been carried out. We have found that other proteins which are not specific to RM, e.g., two proteins of mol wt 15,000 and 50,000, also remain consistently associated with the ribophorins in Kyo EOB residues obtained from RM, as well as in the ribophorin complexes recovered from RM stripped of ribosomes, solubilized with cholate.

Ohlsson and Jergil (63) have reported that considerable amounts of cytochrome P-450 may be recovered in association with polysomes after RM membranes are solubilized with Triton X-100. On this basis, these authors suggested that cytochrome P-450 provides membrane binding sites for at least certain classes of polysomes. It is difficult to see, however, how cytochrome P-450, which is the major protein of rat liver microsomes and is present in both smooth and rough microsomes, could confer ribosome binding specificity to the rough membranes. We found that several

other membrane proteins, including cytochrome P-450 and the NADH and NADPH cytochrome *c* reductases, are incompletely extracted by neutral detergents and are consistently recovered together with the ribophorins in microsomal residues obtained by differential centrifugation. The association of these proteins with the ribosomes, however, does not follow a stoichiometric relationship and is therefore considered unspecific.

Membranes of rough and smooth portions of the ER are known to be continuous within the cell, and several investigators have previously emphasized the existence of striking qualitative biochemical similarities between them. Many enzymatic systems have been shown to be present in both types of membranes, which also have similar phospholipid composition and phospholipid-to-protein ratios (e.g., references 5, 9, 20, 21, 26, 35). Evidence for mechanisms leading to an equilibration in the distribution of membrane proteins in both portions of the endoplasmic reticulum has also been provided (67). Moreover, transport of newly synthesized components along fluid endoplasmic reticulum membranes is thought to be an important biogenetic mechanism, which insures the subcellular distribution of membrane proteins destined for the ER and other membrane systems (cf. references 70, 78). The question should therefore be raised as to how the segregation of ribosome binding sites is maintained intracellularly, so that bound ribosomes are restricted to areas of the ER well demarcated topographically and morphologically.

The number of bound ribosomes per unit area is known to vary within a continuous spectrum in different portions of the endoplasmic reticulum. While in the most densely ribosome-studded areas more than 400 ribosomes/ μm^2 are found (64), pure smooth microsomal vesicles, such as those obtained from phenobarbital-treated animals, bear no ribosomes and are devoid of ribosome-binding sites (60). Correspondingly, RM with different ribosome and ribophorin content (unpublished data with Ms. M. Czako-Graham) and therefore different densities and sedimentation rates are obtained during cell fractionation. These observations indeed suggest that within the cell rather effective structural restraints are imposed on the free diffusion of ribosome-binding sites along the plane of the ER membranes, in spite of the fact that, *in vitro*, considerable lateral displacements of membrane-bound ribosomes within microsomes have been observed and are likely to

occur during protein synthesis (64). Restraints preventing long-range displacements of ribosomes are likely to reflect interactions between ribosome-binding sites, which may serve to stabilize the organization of a ribophorin network. Other investigators (29) have noted that the uneven distribution of ribosomes in the ER membranes may also correspond to lateral enzymatic and compositional heterogeneities in the ER membranes. Some degree of enzymatic and compositional heterogeneity throughout the ER may indeed result from the existence of an extended network of membrane proteins which provides ribosome-binding sites, but also restricts the mobility of other membrane components.

As is reported in the following paper (46),¹ it may be inferred from the accessibility to macromolecular probes, such as proteases and lactoperoxidase, that the ribophorins are exposed on both sides of the ER membranes and that they extend well beyond the limited membrane domains which are covered by the ribosomes. Although a definitive proof is yet lacking, several observations suggest that within the native membranes the ribophorins may be associated with each other forming an intramembranous network which may interconnect the ribosome-binding sites. Using cholate to solubilize the membranes, ribophorin complexes without ribosomes could be isolated from microsomes which had been previously treated with puromycin-KCl. An extended configuration of the ribophorins in the plane of the ER membranes is also suggested by the morphological appearance of microsomal remnants obtained after Kryo EOB treatment. In the electron microscope, these appear as planar lamellae to which ribosomes remain attached or as group of polyosomes to which a filamentous material is attached. That components of the binding sites extend beyond the ribosome-membrane junction may be inferred from the fact that aggregation of ribosome-binding sites also produces the aggregation of most intramembranous particles, as can be seen by freeze fracture (64).

It is now recognized that in spite of the possibility of independent movement of integral membrane proteins in the plane of the lipid bilayer

¹ Rodriguez Boulan, E., D. D. Sabatini, B. N. Pereyra, and G. Kreibich. 1978. Spatial orientation of glycoproteins in membranes of rough microsomes. II. Transmembrane disposition and characterization of glycoproteins. *J. Cell Biol.* In press.

(87), restrictions to the mobility of proteins in membrane systems may be imposed by protein-protein interactions within the membranes or by interactions of membrane proteins with membrane-associated cytoskeleton filamentous elements. The first mechanism may maintain the structural organization of membrane proteins even in the absence of lipids, as is the case of mitochondria (31). The second mechanism may control the mobility of membrane proteins through the degree of polymerization of membrane-associated networks, such as the spectrin network on the cytoplasmic face of erythrocyte membranes (cf. references 88, 92), which is thought to interact with band no. 3, the major integral membrane protein of erythrocytes. Solubilization of membranes with nonionic detergents may in both cases lead to the recovery of protein complexes in which native interactions may be maintained. Indeed, treatment of hepatocyte plasma membranes with Triton X-100 has led to the isolation of gap junctions (36) and an intact network of spectrin and monomeric actin has been recovered from erythrocyte ghosts treated with Triton X-100, as a spherical basket of interconnected filaments (99).

A filamentous network which remains associated with the nuclear pore complexes and retains the spherical shape of the nuclei has been extracted from interphase nuclei treated with nonionic detergents, which remove most other components of the nuclear envelope (1, 2, 10, 75, 82, 42). In this residue, three main polypeptides (69,000, 68,000, and 66,000 daltons) have been recognized which are presumed to be components of the inner nuclear membrane and to play a role in the organization and spacing of the pore (2). Considering the estimated molecular weights of these polypeptides, it appears possible that ribophorins or related polypeptides are present in the nuclear envelope. A structural relationship between the rough ER and the nuclear envelope membranes may also reflect the spatial continuity of both membrane systems (95).

A framework or scaffolding of intrinsic proteins bearing ribosome-binding sites, such as may be provided by the ribophorins, would also appear to be the type of structural feature which could confer to the rough ER membranes their characteristic morphological appearance. In the hepatocyte and in many other cell types, rough ER membranes containing large numbers of ribosomes frequently have the characteristic configuration of flattened sacs or cisterna, arranged in

parallel stacks, which may be retained after cell fractionation (55). The organization of membranes in these arrays contrasts with that of smooth ER membranes which in the form of narrow or tortuous tubules, which appear to branch or vesiculate frequently, may permeate distant portions of the cell or penetrate into the glycogen-containing areas of the cytoplasm (19). A correlation between the presence of ribosomes and the configuration of the underlying ER membranes is also apparent in the morphological appearance of isolated RM, which are usually larger and more homogeneous in size and shape than smooth microsomes. Further evidence for this correlation and for a role of the ribosome-binding sites in determining the correlation and for a role of the ribosome-binding sites in determining the configuration of the membranes is obtained from observations (64) made on isolated RM in which ribosomes on the surface of the vesicles were aggregated experimentally. It was shown that after ribosomes are aggregated in patches, either through cross-linking with antibodies against ribosomal proteins or after ribonuclease treatment of intact microsomes, the appearance of membranes in smooth portions of the microsomal surface differed from that of regions bearing ribosomes. While membrane segments under ribosome patches appeared straight or evenly curved, and therefore had a more rigid appearance, in the same vesicles, membrane portions devoid of ribosomes were thinner and frequently had an undulating appearance or produced blebs and broke into smaller vesicles (64).

Morphological differentiations in the organization of the ER membranes are likely to correspond to functional specializations of rough and smooth portions of the ER in intact liver cells. A protein girdle or filamentous network interconnecting the ribosome-binding sites within the ER membranes, such as the ribophorins, not only would maintain the topographical segregation of ribosomes in rough portions of the ER in spite of the continuity of rough and smooth membranes, but also could contribute to the establishment and maintenance of other local differentiations within the ER system. A structural rigidity imposed by ribophorins to ER membranes bearing ribosomes may explain the electron microscope observation that membrane vesicles or convoluted tubules connecting the ER to other compartments do not originate from ribosome-studded areas or fuse

into rough portions of the ER (40). Instead, images thought to represent the shuttling of membrane vesicles are frequently associated with smooth portions of the ER. Mechanisms regulating the interaction of ribosome-binding sites with one another or with membrane-associated proteins are likely to exist. They may control the mobility of bound ribosomes and determine the presence of local morphological differentiations within the ER.

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