Recovery of Ribophorins and Ribosomes in "Inverted Rough" Vesicles Derived from Rat Liver Rough Microsomes

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ABSTRACT Treatment of rat liver rough microsomes (3.5 mg of protein/ml) with sublytical concentrations (0.08%) of the neutral detergent Triton X-100 caused a lateral displacement of bound ribosomes and the formation of ribosomal aggregates on the microsomal surface. At slightly higher detergent concentrations (0.12–0.16%) membrane areas bearing ribosomal aggregates invaginated into the microsomal lumen and separated from the rest of the membrane. Two distinct classes of vesicles could be isolated by density gradient centrifugation from microsomes treated with 0.16% Triton X-100: one with ribosomes bound to the inner membrane surfaces ("inverted rough" vesicles) and another with no ribosomes attached to the membranes. Analysis of the fractions showed that ~30% of the phospholipids and 20–30% of the total membrane protein were released from the membranes by this treatment. Labeling with avidin-ferritin conjugates demonstrated that concanavalin A binding sites, which in native rough microsomes are found in the luminal face of the membranes, were present on the outer surface of the inverted rough vesicles. Freeze-fracture electron microscopy showed that both fracture faces had similar concentrations of intramembrane particles. SDS PAGE analysis of the two vesicle subfractions demonstrated that, of all the integral microsomal membrane proteins, only ribophorins I and II were found exclusively in the inverted rough vesicles bearing ribosomes. These observations are consistent with the proposal that ribophorins are associated with the ribosomal binding sites characteristic of rough microsomal membranes.

Rough endoplasmic reticulum membranes play an important role in protein biosynthesis by providing sites for the binding of ribosomes involved in the synthesis of specific classes of polypeptides that are transferred into the lumen of the endoplasmic reticulum (ER) or inserted into the membrane (1–3). ER membrane proteins also participate in ordered sequences of co- and post-translational modifications of polypeptides synthesized on bound polysomes, including proteolytic processing (4–7), glycosylation (8), formation of disulfide bonds (9), and modifications of amino acid side chains (10, 11). There is, therefore, considerable interest in relating the different functions of rough microsomes (RM) in protein biosynthesis to specific protein components of the ER membranes.

Comparative electrophoretic analysis of purified rough and smooth microsomes obtained from rat liver and several other sources has revealed characteristic differences in the protein composition of membranes derived from rough and smooth portions of the ER (12–15), which may reflect their functional specialization. RM contain two glycoproteins (65,000 and 63,000 mol wt), which have been designated ribophorins I and II and are absent from smooth microsomes (SM). A relationship between these proteins and the binding sites for ribosomes in the rough ER was suggested by the observation that the ribophorin content of microsomes of different isopycnic density is stoichiometrically related to the number of ribosomes and by the finding that ribophorins can be recovered in association with sedimentable ribosomes after most other microsomal proteins are solubilized by treatment with certain nonionic detergents.

In this paper we demonstrate that treatment of RM with concentrations of Triton X-100 that do not cause complete lysis of the membranes leads to extensive aggregation of bound

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ribosomes on the microsomal surface and to a concomitant change in the configuration of the vesicles. These observations led us to develop a procedure for the subfractionation of RM into two types of vesicles derived from different regions of the rough microsomal membranes. One of the microsomal subfractions consists of inverted rough vesicles containing ribophorins and bound ribosomes, while the other contains vesicles that seem to be derived from areas of rough membranes devoid of both bound ribosomes and ribophorins.

The concomitant redistribution of ribophorins and ribosomes that occurs during the subfractionation of rough microsomes lends support to the proposal that ribophorins are associated with ribosome binding sites in the rough membranes and reinforces the suggestion that these proteins may play a role in the vectorial discharge of nascent polypeptides into the ER lumen or in their insertion into the membranes.

MATERIALS AND METHODS

General

All solutions were prepared with double-distilled water, were Millipore-filtered (0.45-μm pore size for most, 1.2-μm for concentrated sucrose and acrylamide solutions) and stored at 4°C. The notation Tris will be used for Tris-HCl, pH 7.4 at 4°C.

Unless otherwise specified, all centrifugations were carried out at 3°C using Beckman rotors and ultracentrifuges (L3-50, L5-50, or L5-65; Beckman Instruments, Palo Alto, CA). An abbreviated notation is used to specify centrifugation conditions, e.g., 30 min/30k/Ti60, stands for centrifugation at 30,000 rpm in a Ti60 rotor for 30 min at 3°C.

The following buffer solutions were used: high salt buffer (HSB): 500 mM KCl, 50 mM Tris, 5 mM $MgCl_2$; low salt buffer (LSB): 50 mM KCl, 50 mM Tris, 5 mM $MgCl_2$; TKM: 25 mM KCl, 50 mM Tris, 5 mM $MgCl_2$. When sucrose was included, its concentration is indicated first, e.g., 10% SLSB indicates low salt buffer containing 10% sucrose.

Reagents

Chemicals were purchased from the following sources: Trizma base (Tris), dithiothreitol, triethanolamine (TEA), SDS, NADH, and NADPH from Sigma Chemical Co. (St. Louis, MO). Coomassie Brilliant Blue and enzyme grade sucrose from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY); deoxycholic acid from Schwartz/Mann Div., *N,N'*-methylenebisacrylamide, acrylamide, Triton X-100, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) and butylhydroxy toluene (BHT), from Eastman Kodak Co. (Rochester, NY); salts in buffer solutions were analytical grade reagents from Fisher Scientific Company (Springfield, NJ). Ultrapure glutaraldehyde was purchased from Tousimis Research Corp. (Rockville, MD) and [^{3}H]choline hydrochloride from New England Nuclear (Boston, MA).

The nonionic detergent Kyro EOB (a polyethoxyalkylether) was a gift from Dr. H. Hughes (Miami Valley Research Laboratory, Procter and Gamble Co., Cincinnati, OH).

Preparation of Microsomal Fractions

Unless specifically mentioned, 150-g Sprague-Dawley male rats starved overnight before sacrifice were used to prepare RM, by a modification of the procedures of Adelman et al. (16) and Kruppa and Sabatini (17). Microsomal membranes containing labeled phospholipids were isolated from rats that received an intraperitoneal injection of 200 μCi of methyl-[^{3}H]choline (sp act, 80 Ci/mmol) 4 h before sacrifice. The labeled RM contained ~9 × 10^4 dpm/mg of microsomal protein.

Analytical Procedures

Protein concentration was measured in duplicate aliquots using the method of Lowry et al. (18) and bovine serum albumin (BSA) as a standard. The BSA concentration was determined from its optical density at 279 nm according to Foster et al. (19). The RNA content of microsomes was estimated from the OD_{260} after treatment with 1% SDS (ribosome $E^{1\%}_{260}$ = 135; Tashiro and Siekevitz, [20]) or measured by the procedure of Fleck and Munro (21). Phospholipids (PL) were extracted according to Folch et al. (22) and the phosphorus concentration was measured according to Ames and Dubin (23). 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 (24) and Omura et al. (25), using an Aminco DW-2 spectrophotometer (American Instruments, Silver Spring, MD).

Sucrose Density Gradient Centrifugation

Composition of the gradients and conditions of centrifugation are given in figure legends. Absorbance profiles at 254 nm after sucrose density gradient centrifugation were monitored with an auto-densiflow probe (Buchler Instruments, Fort Lee, NJ) connected to an LKB Uvicord II monitor and a Perpex periplastic pump (LKB, Bromma, Sweden) and recorded in a Hewlett-Packard linear recorder with a log converter. The radioactivity distribution was measured in fractions collected from effluent of the system at 10-s time intervals.

PAGE

Discontinuous SDS PAGE 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 (26). Aside from minor modifications, all buffer and solutions were those described by Maizel (27; see also Kreibich and Sabatini [28]).

Electrophoresis was carried out at 15–25 mA until the tracking dye was one-half 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. Rabbit myosin (210,000), β-galactosidase (130,000), bovine serum albumin (68,000), hen ovalbumin (43,000), pig urate oxidase (34,000), calf chymotrypsinogen (25,000), and rabbit globin (14,800) were used as molecular weight standards for the calibration of SDS acrylamide gels.

Electron Microscopy

Microsomes or microsomal subfractions were fixed in suspension with 2% glutaraldehyde maintaining the final detergent concentration. After 30–60 min the sedimentable fractions were recovered (30 min/30k/SW56) as pellets, which were rinsed, postfixed with OsO_4, stained *en bloc* with uranyl acetate, and embedded in Epon. Thin sections were poststained with lead acetate.

Freeze-fracture replicas of the microsomal subfractions were prepared as previously described (29). The localization of lectin binding sites in the membranes was established by the procedure of Rodriguez-Boulan et al. (30), using biotinized concanavalin A (Con A) and ferritin-avidin conjugate (31).

RESULTS

We have previously demonstrated (29) that membrane-bound ribosomes and the binding sites to which they are attached in the ER membranes can, under certain conditions, undergo extensive lateral displacement in the plane of microsomal membranes and form aggregates in localized regions of the microsomal surface. This was observed, for example, when microsomes treated with low concentrations of ribonuclease or antibodies against ribosomal proteins were incubated at temperatures above that which causes a phase transition in the membrane phospholipids. Electron microscope observations showed that treatment of RM with low concentrations of the neutral detergents Triton X-100 (Fig. 1 *b* and *c*) and Kyro EOB (Fig. 2 *a–c*) also leads to the formation of aggregates of bound ribosomes on the microsomal surface without causing their detachment. Ribosome aggregation was observed in microsomes fixed after incubation with 0.08% Triton X-100 (Fig. 1 *b*) or with 0.12% and 0.25% Kyro EOB (Fig. 2 *a* and *b*). These detergent concentrations have been shown to cause negligible solubilization of microsomal phospholipids and to release mainly proteins contained within the microsomal lumen (32, 33). Characteristically, microsomes treated with low concentrations of Kyro EOB (0.12 or 0.25%) remained as rounded vesicles exhibiting regions of extensive ribosome aggregation and areas free of ribosomes (Fig. 2 *a* and *b*). On the other hand, the segregation of ribosomes into localized membrane domains produced by treatment with Triton X-100 (Fig. 1 *b*) was accom-

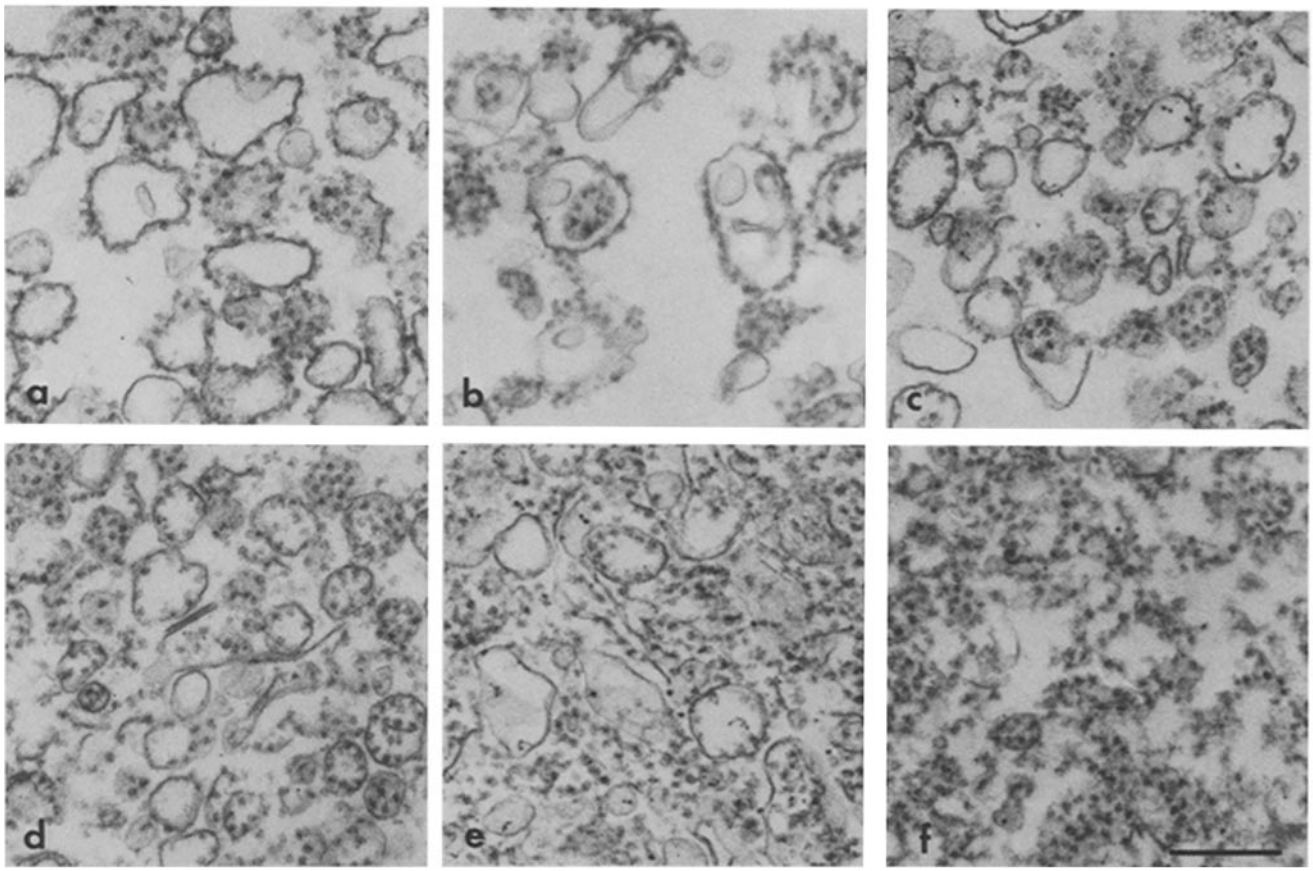

FIGURE 1 Effect of increasing concentrations of Triton X-100 on rat liver microsomes. Rat liver RM stored in glycerol (60%) at −70°C were diluted three times with a low salt buffer (LSB) containing 50 mM TEA, pH 7.6, instead of Tris, and washed by centrifugation (30 min, 100,000 *g*). The pellets were resuspended in LSB (3.5 mg protein/ml), and to 0.54-ml aliquots 60 μl of a Triton X-100 solution was slowly added while stirring on a Vortex, so that the final detergent (Triton X-100) concentration was (*b*) 0.08%, 1.3×10^{-3} M; (*c*) 0.12%, 2×10^{-3} M; (*d*) 0.16%, 2.6×10^{-3} M; (*e*) 0.20%, 3.3×10^{-3} M; or (*f*) 0.24%, 3.9×10^{-3} M. A control sample (*a*) received 60 μl of water instead of detergent. The incubation mixtures were kept for 30 min at 4°C and aliquots were fixed with 2% glutaraldehyde for 2 h at 0°C. After samples were diluted with LSB and sedimented (15 min, 30,000 *g* on an SW56 rotor), the pellets were prepared for electron microscopy. Samples treated with 0.08% Triton X-100 (*b*) showed extensive aggregation of ribosomes on invaginated surfaces, whereas microsomes treated with 0.16% Triton X-100 (*d*) fragmented into rough inverted and smooth surfaced vesicles. Higher Triton X-100 concentrations (*e* and *f*) cause progressive disassembly of the membranes. Bar, 0.2 μm. × 62,000.

panied by striking changes in microsomal morphology. Many vesicles showed membrane invaginations bearing ribosomal aggregates, as well as ribosomal aggregates on the outermost membrane surface.

To compare the composition of membrane domains with or without ribosome binding sites, we attempted to establish conditions for the subfractionation of microsomes carrying ribosome aggregates into vesicles derived from the ribosome-bearing and the ribosome-free areas. As has been previously described (12), high concentrations of Kyro EOB (0.5–1%) caused extensive phospholipid and protein solubilization yielding curved membrane remnants to the convex side of which ribosomes remained attached (Fig. 2*d*). The effect of a series of Triton X-100 concentrations, ranging from 0.08 to 0.24%, on the phospholipid and protein content of the microsomal membranes was assessed by sucrose density gradient analysis of microsomes labeled in vivo with [^{3}H]choline (Fig. 3) and by SDS protein electrophoresis of supernatant and sedimentable fractions obtained by differential centrifugation (Fig. 4). It was found that release of [^{3}H]choline-labeled phospholipids started at 0.12% Triton X-100 (Fig. 3*c*) and increased with the detergent concentration. At 0.16% Triton X-100, ~30% of the microsomal phospholipids were solubilized (Fig. 3*d*) and substantial amounts of cytochrome P-450 and other membrane proteins were released into the supernatant fractions (Fig. 4*d*). At 0.25% and higher Triton X-100 concentrations, the solubilization process was almost completed, and the upper fractions of the gradients contained >80% of the phospholipids (Fig. 3*f*) and, except for the ribophorins, a full complement of all other microsomal proteins (Fig. 4*e*). At these concentrations, only polysomal aggregates containing the ribophorins and ~30% of the microsomal phospholipid sedimented within the gradients, producing polysome-like patterns (Fig. 3*f*). The association of the ribophorins with the sedimentable ribosomes was disrupted only at levels of Triton X-100 >0.24% (Fig. 4*f*). Electron microscope analysis of the Triton-treated RM showed that, at the 0.16% detergent level (Fig. 1*d*), microsomes gave rise to two main classes of vesicles; one with rounded profiles and ribosomes bound to their inner surfaces, and another consisting only of smooth vesicles. It appears reasonable to assume that the first type originated from the pinching off of membrane invaginations (Fig. 1*b*), which became more abundant as the concentration of Triton approached 0.16%, while the second was formed by the resealing into vesicles of the remaining areas

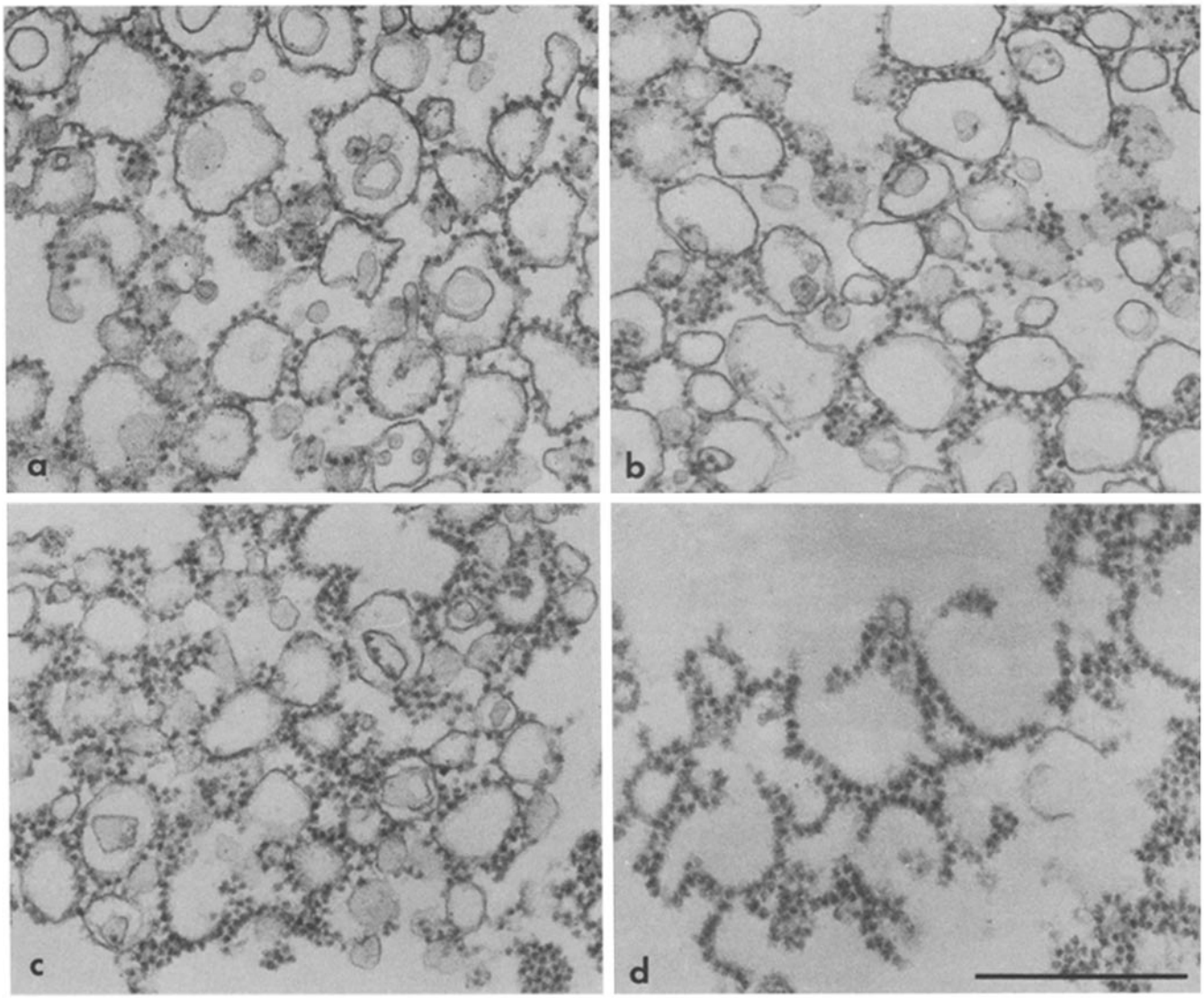

FIGURE 2 Aggregation of ribosomes on convex surfaces of rough microsomes treated with the nonionic detergent Kyro EOB. Washed RM resuspended in LSB (for details, see legend to Fig. 1) were incubated with increasing concentrations of Kyro EOB: (*a*) 0.12%, 3×10^{-3} M; (*b*) 0.25%, 6×10^{-3} M; (*c*) 0.5%, 1.25×10^{-2} M; and (*d*) 1%, 2.5×10^{-2} M. Sediments obtained after fixation with glutaraldehyde (2% final) were prepared for electron microscopy. Low concentrations of Kyro EOB (*a* and *b*) caused aggregation of ribosomes into polar caps which at higher detergent concentrations (*c* and *d*) remained associated with the convex faces of microsomal membrane remnants. Bar, 0.5 μm. × 75,000.

of smooth membranes. Concentrations of Triton X-100 >0.16% (Fig. 1 *e* and *f*) produced extensive membrane dissolution and release of bound polysomes.

The differences in ribosome content of the two types of vesicles generated by treatment with 0.16% Triton X-100 suggested that distinct vesicle populations could be separated on the basis of their different isopycnic density. This was accomplished by centrifugation of microsomes treated with 0.16% Triton X-100, through a gradient ranging in sucrose concentration from 20 to 60% (upper half of Fig. 5). After centrifugation for 16 h, fractions (I–VI) were collected, sedimented after dilution, and analyzed chemically (Table I), by SDS gel electrophoresis (lower half of Fig. 5), and by electron microscopy (Fig. 6). The results given in Fig. 6 and in Table I indicate that ribosomes were recovered mainly in two fractions (V and VI), which had a phospholipid-to-protein ratio (0.3–0.2) lower than that of the original microsomes (1.1) but contained 85% of the total RNA and higher RNA/protein ratio than control microsomes (0.18). Electron microsopy showed that fraction VI (Fig. 6 *e*) contained a large proportion of rough inverted vesicles while rough vesicles of normal sideness predominated in fraction V (Fig. 6 *d*). Vesicles of mixed character with a nearly normal RNA/protein ratio (0.194) and partially studded with ribosomes were recovered in fraction IV. Smooth vesicles derived from the RM by the detergent treatment, which had a high phospholipid-to-protein ratio (0.6–0.5), were recovered largely in fractions of lower density (II, III) (Fig. 6 *a* and *b*). The low ribosome content of these vesicles is reflected in RNA/protein ratios, which ranged from 0.02 to 0.08. A small sediment of very dense material containing exclusively rough inverted membrane residues of low phospholipid content (phospholipid/protein ratio, 0.071) was always recovered after treatment with 0.16% Triton X-100 (Fig. 6 *f*). Electrophoretic analysis (lower half of Fig. 5) showed that the ribophorin content of the different fractions increased in parallel with the RNA/protein ratio and the density of the vesicles.

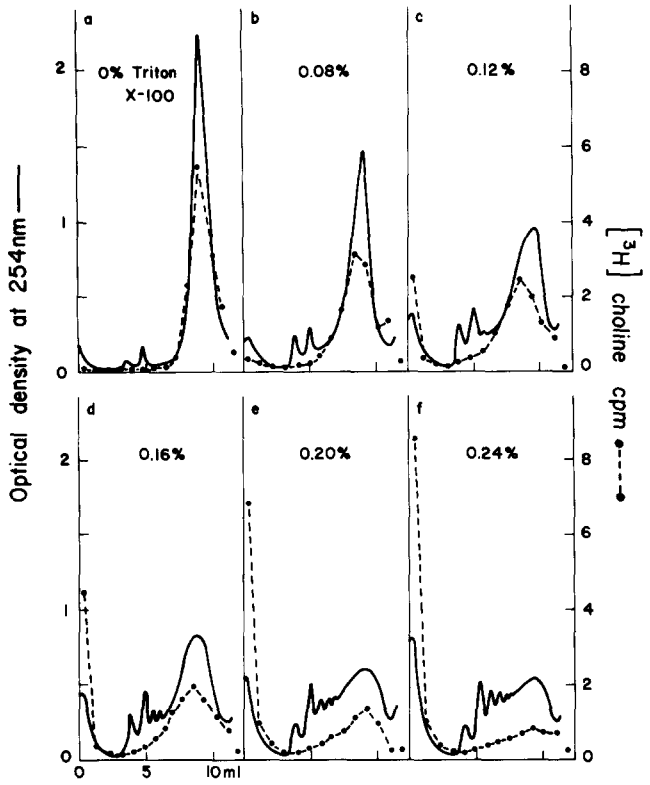

FIGURE 3 Release of phospholipids from rough microsomes at various concentrations of Triton X-100. Rough microsomes labeled in the phospholipid moieties were prepared from rats injected with [^{3}H]choline and treated with increasing concentrations of Triton X-100 (for details, see Materials and Methods and legend to Fig. 1). 400-μl aliquots from each incubation mixture were loaded onto linear sucrose density gradients (10–60%) containing LSB. After centrifugation (2 h, 200,000 *g* on an SW41 rotor), the optical density throughout the gradient was recorded and the distribution of ^{3}H-labeled phosphatidyl choline was measured in 0.5-ml subfractions. At Triton X-100 concentrations <0.12% (*a–c*), all the phospholipid radioactivity was found with the membrane peak, whereas at 0.24% (*f*) the detergent released >80% of the labeled phospholipids, which were found near the top of the gradient. Microsomes treated with 0.16% (*d*) Triton X-100 retained 70% of the labeled phospholipids, which were recovered with the sedimentable fraction of vesicles.

A preparative method to obtain rough inverted vesicles in amounts suitable for further analysis was developed on the basis of the preceding observations. RM treated with 0.16% Triton X-100, a concentration that produced the highest yield of rough inverted vesicles (Fig. 1 *d*), were subfractionated by sedimentation through a discontinuous density gradient consisting of two layers of 30 and 10% sucrose. A population of rough inverted vesicles was recovered from the bottom of the tubes (Fig. 7), while a fraction of smooth vesicles remained within the 10% sucrose layer (Fig. 7, *inset*). Electrophoretic analysis (Fig. 8) showed that solubilized microsomal content proteins as well as a small proportion of membrane proteins were recovered in the loading region (Fig. 8 *d*).

An analysis of the rough inverted fraction (Table II) showed that it approximated fraction VI (Fig. 5 *VI*, Fig. 6 *e*, and Table I) in composition. The complete retention of ribophorins and ribosomes in this fraction, which is apparent from the electro-

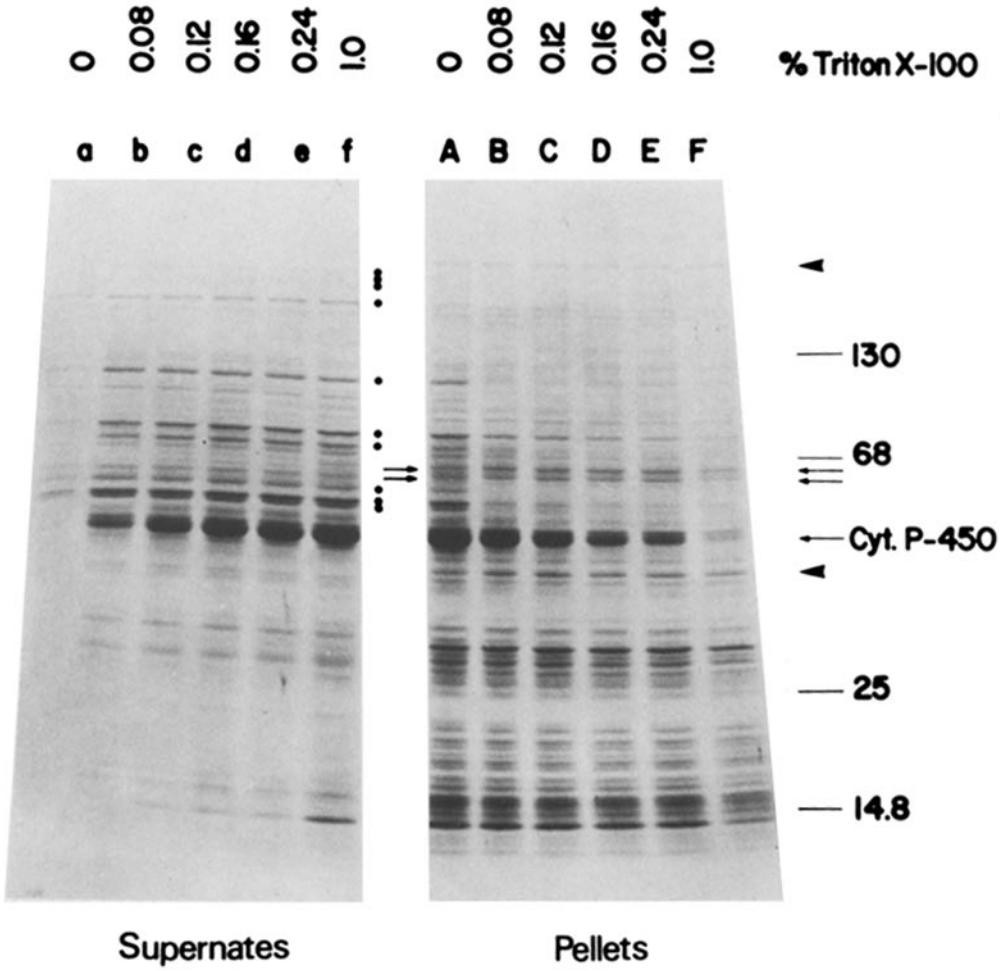

FIGURE 4 Subfractionation of RM treated with different concentrations of Triton X-100. Washed RM resuspended in LSB (see legend to Fig. 1) were incubated with the different concentrations of Triton X-100 indicated in the figure. 1.5-ml samples were centrifuged (60 min, 100,000 *g*), and the pellets were resuspended in the original volume. Aliquots of both the resuspended pellets (*A–F*) and supernatants (*a–f*) (150-μl each) were removed for SDS PAGE on 6–11% gradient gels. Positions of the two ribophorins and cytochrome P-450 in the gels are indicated by arrows. Microsomal content proteins (32) in track *f* are labeled with dots. As can be seen from the Coomassie Blue staining patterns, at Triton X-100 concentrations <0.24% both ribophorins remained associated with the sedimentable fractions. Arrowheads mark the positions of myosin (20 kdaltons) and actin (45 kdaltons) that are present in RM preparations and sediment to the pellet after detergent treatment. Molecular masses are given at the right of the figure in kdaltons.

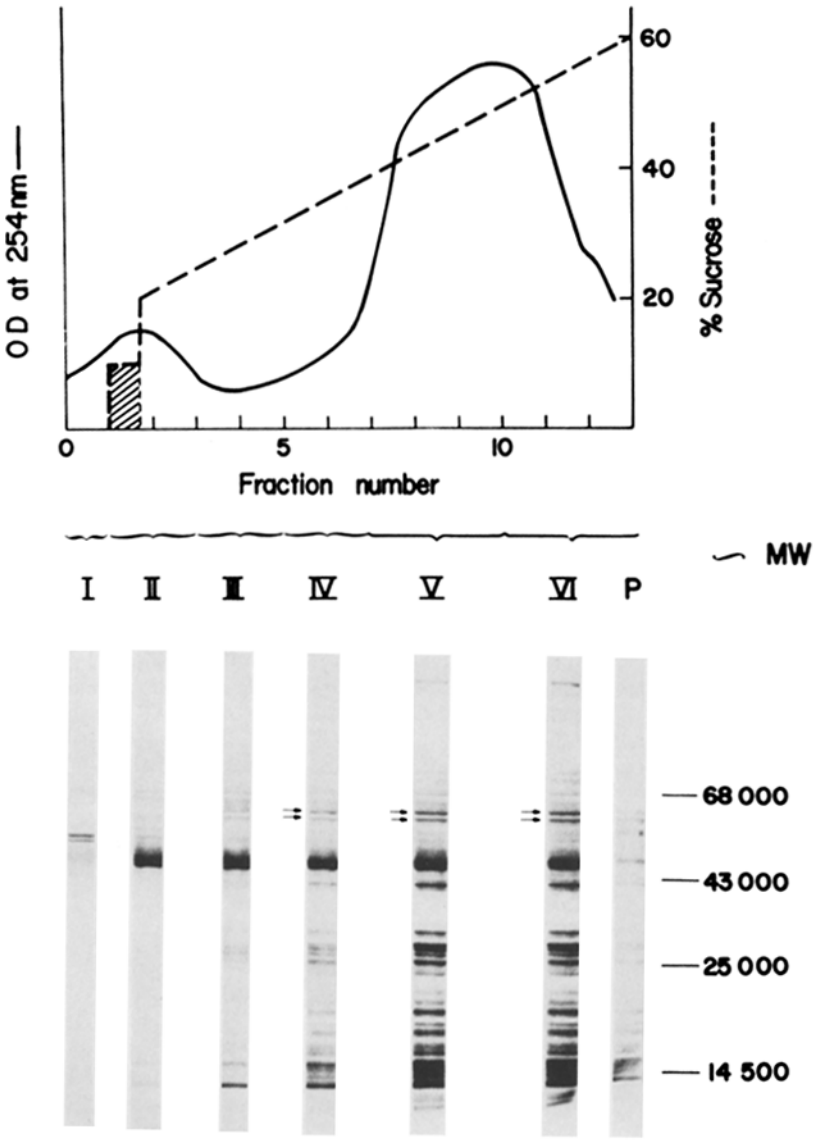

FIGURE 5 Subfractionation of RM treated with 0.16% Triton X-100 by isopycnic centrifugation. A suspension of RM (3.5 mg of protein/ml) was treated with Triton X-100 (final concentration, 0.16%). After incubation for 30 min at 3°C, 9-ml samples were loaded onto linear sucrose gradients (20–60% SLSB, overlaid with 2 ml of 10% SLSB containing 0.16% Triton X-100), which had been prepared in tubes for the SW27 Spinco rotor. After centrifugation for 16 h (20,000 *g* on an SW27 rotor), the OD profile was recorded and 3-ml fractions were collected and pooled (*I–VI*) as indicated in the figure. Fractions II–VI were diluted three times with LSB. The pellet (*P*) and the sediments obtained from the fractions after centrifugation (60 min, 40,000 *g* on a Ti60 rotor) were suspended in 1 ml of LSB. Samples were used for biochemical analysis (Table I) or thin-section electron microscopy (Fig. 6) after removing aliquots (150 μl of fractions II–IV and 100 μl of fractions V, VI, and P) for SDS acrylamide (6–11%) gel electrophoresis. The position of the two ribophorins in the gels is indicated by arrows. The intensity of the ribophorin bands increases in parallel with the content of ribosomal proteins (bands in the lower half of the gels).

TABLE I

Biochemical Analysis of Rough Microsomal Subfractions Obtained after Treatment of RM with 0.16% Triton X-100 *

Gradient fraction	Protein	P-Lipid	RNA	RNA/protein	P-Lipid/protein	$OD_{260/280}$
	mg	*mg*	*mg*			
I	10.2	3.70	0.12	0.011	0.332	0.733
II	4.8	2.91	0.10	0.02	0.604	1.04
III	2.6	1.42	0.21	0.08	0.538	1.26
IV	1.7	0.72	0.33	0.194	0.412	1.64
V	8.5	2.8	3.19	0.374	0.329	1.70
VI	3.8	0.81	1.53	0.407	0.211	1.72
P	0.3	0.02	0.25	0.840	0.071	1.83

* Subfractions of different isopycnic densities were obtained as described in the legend to Fig. 5 and analyzed for their protein, lipid, and RNA content (for details, see Materials and Methods).

phoretic pattern (Fig. 8*b*), contrasts with the behavior of other integral membrane proteins, such as cytochrome P-450, its reductase, and cytochrome b_5, which were found in both sedimentable subfractions produced by the detergent treatment (Table II). The extent of ribosome release caused by puromycin in a medium of high salt (34) was used to estimate the proportion of RM inverted by the detergent treatment (see Table III).

It has previously been shown that detergent treatment does not prevent dissociation of ribosomes caused by puromycin. It was found that ~18% of the ribosomes remained associated with the membranes of control RM, but 61–69% of the ribosomes sedimented with the membranes after this treatment. Assuming that no inverted vesicles are opened by the treatment with puromycin in a high salt medium, this indicates that at least 50% of the vesicles are in the inverted conformation. Studies of the proportion of inverted vesicles made from electron micrographs indicated that in the best preparations up to 80% of the rough microsomes were in the configuration (Fig. 7). The yield of inverted vesicles appeared to be unaffected by changes in the Mg^{++} (0–10 mM) and K^+ (25–250 mM) concentrations.

It was of interest to determine whether the asymmetric disposition of proteins in the microsomal membrane was maintained during the process that led to the formation of invaginations and production of inverted vesicles. That this might be the case, in spite of extensive extraction of phospholipids and membrane proteins, was suggested by the association of the ribosomes with only one side of the vesicles. In previous studies on the distribution of lectin binding sites in native rat liver RM, we demonstrated that the carbohydrate moieties of membrane glycoproteins are exposed exclusively in the luminal side

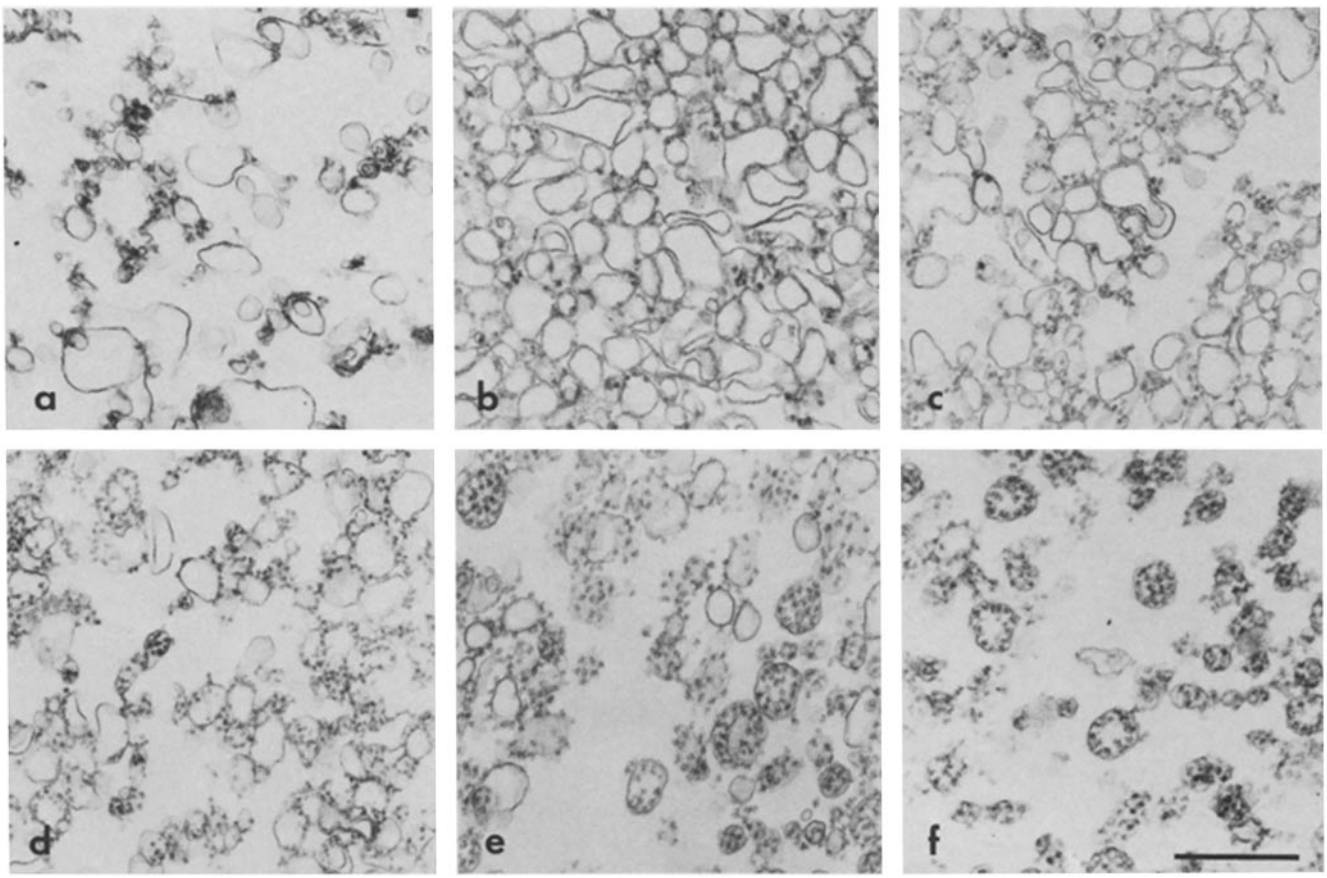

FIGURE 6 Morphological characteristics of subfractions of RM obtained after treatment with 0.16% Triton X-100. RM treated with 0.16% Triton X-100 were subfractionated by isopycnic sedimentation in sucrose density gradients described in the legend to Fig. 5. Sediments obtained from fractions II–VI or the resuspended pellet were fixed in glutaraldehyde (2% final) and prepared for thin-section electron microscopy. Fractions II and III (*a* and *b*) contain predominantly smooth-surfaced vesicles and fractions IV and V (*c* and *d*) contain vesicles partially studded with ribosomes. In contrast, fractions VI and P (*e* and *f*) show vesicles with large numbers of ribosomes, most of which are bound to the luminal faces of the membranes. Bar, 0.5 μm. × 33,000.

of the membranes (30, 35). In inverted vesicles, a topologically equivalent orientation of the glycoproteins would correspond to an exposure of sugar moieties on the outer surface. This was indeed found to be the case when the distribution of biotinized Con A was assessed through the binding of avidin-ferritin complexes, which were visualized by electron microscopy (Fig. 9).

Occasionally, inverted RM with apparent discontinuities in the vesicular membranes were seen (see *inset* in Fig. 9*b*). In these cases, too, only membrane faces opposite the ribosome-bearing sides appeared labeled with ferritin. These observations indicate that the spatial distribution of ribosome binding sites and carbohydrates of membrane glycoproteins on opposite sides of the membranes is preserved through the detergent extraction and inversion procedure. It remains to be seen, however, whether the asymmetric distribution of rough microsomal membrane proteins carrying no carbohydrate chains is also preserved.

It was somewhat surprising to find by freeze-fracture electron microscopy (Fig. 10) that both convex and concave fracture faces of rough inverted vesicles displayed similar intramembrane particle densities, in contrast to the situation with native rough microsomes in which the intramembrane particle partition ratio favors the ribosome-bearing half of the membranes (P-fracture face) (29). It should be noted, however, that a change in partition ratio (the even distribution of particles in both fracture faces) need not reflect a loss in the general asymmetric disposition of the transmembrane microsomal proteins which are thought to be represented by the intramembrane particles. Indeed, the removal of peripheral proteins from the cytoplasmic surface, to which the intramembrane particles may be normally linked, could also change the partition of intramembrane particles during freeze-fracture.

DISCUSSION

We have examined the effect of a series of concentrations of the neutral detergents Triton X-100 and Kyro EOB on rat liver rough microsomes and found that increasing levels of these detergents produce characteristic changes in the distribution of ribosomes, which alter the lateral organization of membrane components and affect differentially the geometry of the vesicles (see Fig. 11). From the sequence of changes observed, an association between the membrane-bound ribosomes and ribophorins I and II, two glycoproteins in the native microsomal membranes, can be inferred. Low concentrations of Triton X-100 (0.08%), which cause leakage of content proteins before substantial solubilization of phospholipid and membrane proteins occurs (32, 33), led to the formation of ribosomal aggregates on the microsomal surface and to the development of invaginations in which these aggregates were collected. Only slightly higher concentrations of the detergent (0.16%), which

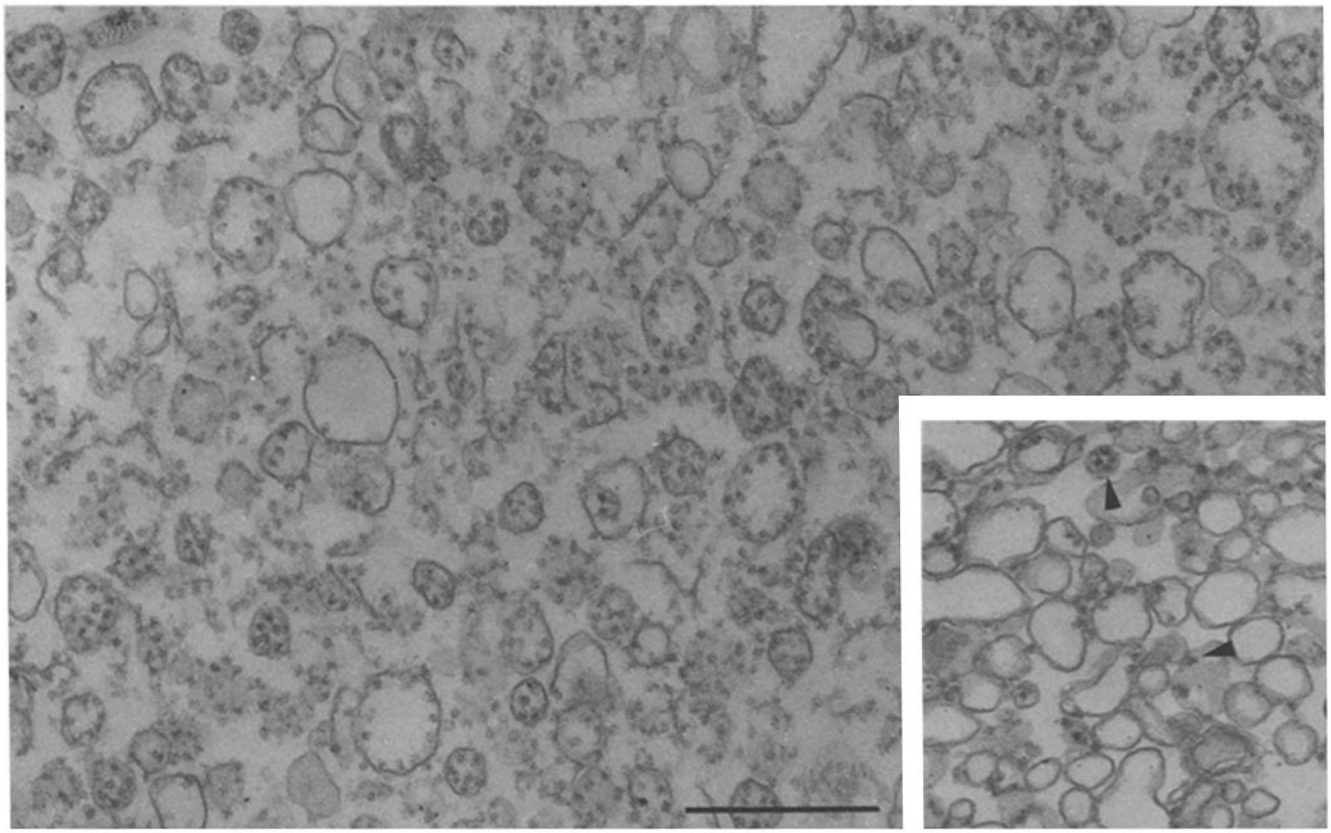

FIGURE 7 Preparative isolation of "inverted rough" vesicles. A suspension of RM (3.5 mg/ml, 2 ml total) treated with 0.16% Triton X-100 (see legend to Fig. 5) was layered on top of a step gradient made of 2 ml of 30% SLSB and 1.5 ml of 10% SLSB. After centrifugation (60 min, 100,000 *g* on an SW56 rotor), the pellet, the band recovered at the interface between the 10 and 30% sucrose layers, and the material found in the loading zone were collected separately. Aliquots of the suspended pellet containing mainly inverted rough vesicles and of the interface fraction (*inset*) were fixed with glutaraldehyde (2% final) and prepared for thin-section electron microscopy. Arrowheads in the *inset* point to a few ribosomes which remain attached to the largely smooth-surfaced vesicles. Bar, 0.4 μm. × 65,000.

TABLE II

Biochemical Characterization of "Rough Inverted" and "Smooth Vesicles" Obtained from RM Treated with 0.16% Triton X-100 *

	Control RM	Solubilized proteins	Smooth vesicles	Rough inverted vesicles	Recovery
					mg (%)
$OD_{260/280}$	1.57	0.78	100	1.76	
Protein, *mg*	3.07	0.67	0.46	1.52	2.65 (86)
RNA, *mg*	0.68	0.11	0.05	0.66	0.82 (120)
Phospholipid, mg/ml	1.12	0.40	0.28	0.36	1.04 (93)
RNA/protein	0.183	0.164	0.108	0.423	—
Lipid/protein	0.364	0.597	0.608	0.230	—
Cytochrome P-450, μg	103	31	16	42	89 (86)
Cytochrome b_5, μg	38	5	11	20	36 (95)
NADPH cytochrome P-450 reductase, μg	12	6	2	3	11 (92)

* RM suspended in LSB and treated with 0.16% Triton X-100 were subfractionated into rough inverted vesicles, smooth-surfaced vesicles, and soluble proteins as described in the legends to Figs. 6 and 7. Values given are averages from two independent experiments. In each case, individual values were within 10% of the average.

began to cause solubilization of membrane components, resulted in the fragmentation of the microsomes into two types of vesicles: inverted rough, which appear to be derived from the invaginated membranes, and smooth vesicles, in which areas of membrane not associated with ribosomes seem to be segregated. At this stage, ribophorins were quantitatively recovered with the inverted vesicle fraction and were the only major membrane proteins found exclusively in this subfraction. Even higher concentrations of Triton X-100 (0.24%) led to the progressive solubilization of the membranes and in turn to the separation of ribophorins from the sedimentable polysomes.

It is interesting to note that a different sequence of events took place when Kyro EOB was used. Although low concentrations of the detergent (0.12 and 0.25%) also produced aggregation of ribosomes, this was not accompanied by the formation of invaginations. Higher concentrations of the detergent (0.5

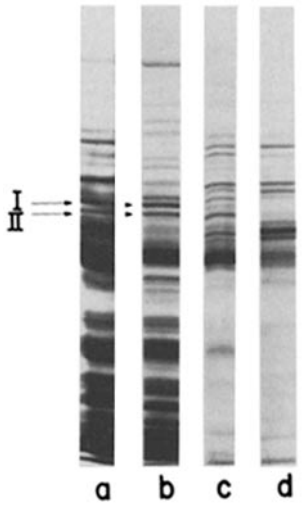

FIGURE 8 Recovery of ribophorins and ribosomes in inverted rough vesicles. RM treated with 0.16% Triton X-100 were subfractionated on a step gradient as described in the legend to Fig. 7. Samples of native RM (200 μg) (*a*) and of the inverted rough (110 μg) (*b*) and smooth-surfaced vesicles (40 μg) (*c*) shown in Fig. 7, as well as 50 μg of the material recovered from the loading zone (*d*), were analyzed on SDS acrylamide gels (6–11%). Arrows and arrowheads indicate the position of the ribophorins which are only found in native RM (*a*) and in the inverted rough fraction (*b*). The supernatant fraction (*d*) consists mainly of content proteins, whereas the smooth-surfaced vesicles (*c*) contain membrane proteins and some content proteins. A Coomassie Blue-staining band observed in lane *c* that migrates with the same mobility as ribophorin II does not react with a goat anti-rat ribophorin II antibody.

and 1.0%) did not alter the configuration of membrane regions bearing ribosomal aggregates, even though most proteins other than the ribophorins were progressively extracted from the membranes. The final result of Kyro EOB extraction is the formation of stable curved membrane remnants that contain ribophorins and bear ribosomes on their convex faces.

The inability of Kyro EOB to solubilize ribophorins at any concentration suggests that this detergent does not interact directly with these proteins. It has been suggested (13, 14) that the presence of a ribophorin network, limited to the rough ER, is responsible for the characteristic morphological features of rough ER cisternae, which form stacks of flattened disks within the cell, and for the organization of ribosome binding sites in rosettes, spirals, and other configurations of bound polysomes (cf. reference 2). The rigid and extended morphological appearance of the membrane areas bearing ribosomes in the remnants obtained after Kyro EOB may also reflect the local high concentrations of these proteins.

The ability of Triton X-100 to cause, at sufficiently high concentrations, complete solubilization of ribophorins suggests that, in contrast to Kyro EOB, this detergent interacts directly with the proteins. An initial association of the detergent with

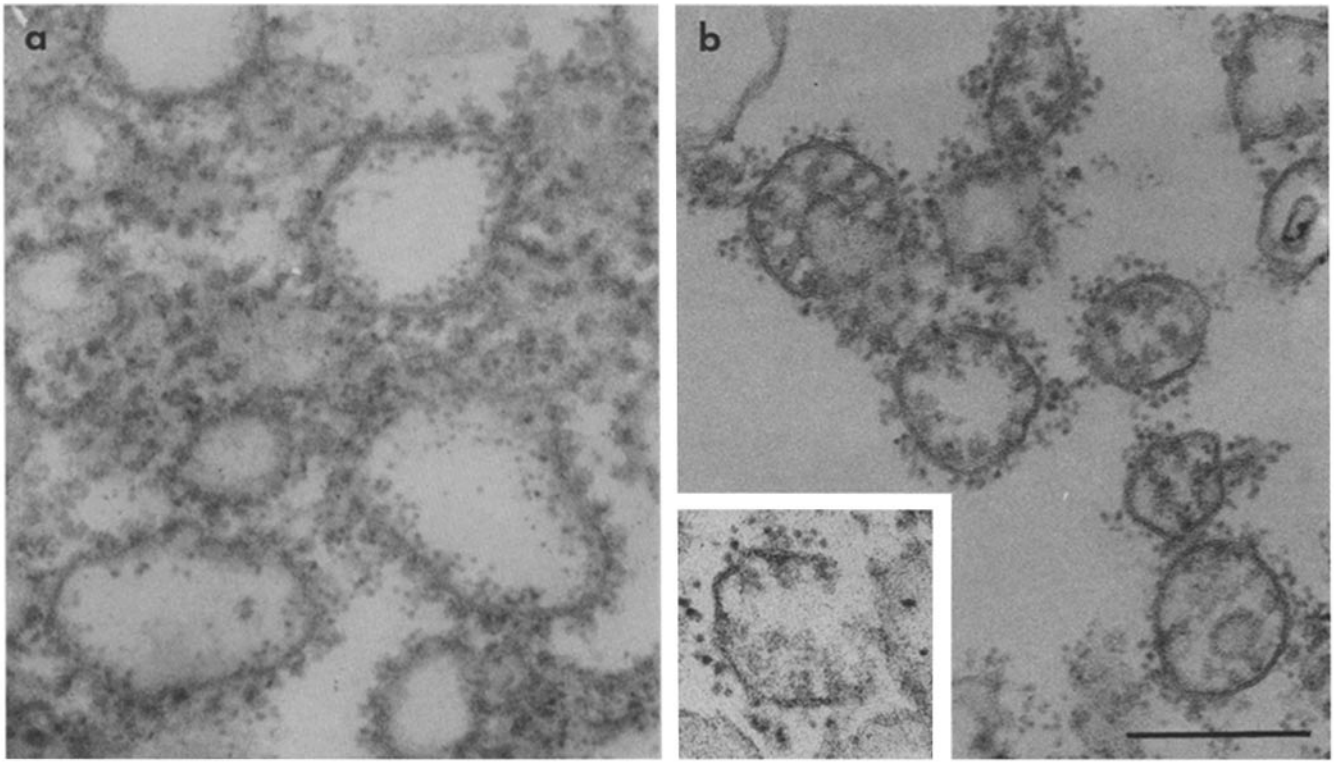

FIGURE 9 Con A binding sites of microsomal glycoproteins are exposed in rough inverted vesicles. Rough inverted vesicles (*b*) prepared as described in the legend to Fig. 7 were treated with biotinized Con A and ferritin-avidin as previously described (30). A sample of control rough microsomes (*a*) from which content proteins had been released by treatment with 0.02% deoxycholic acid (DOC) was fixed in 0.01% glutaraldehyde and then treated with 0.05% DOC to render the vesicle membrane permeable to the biotinized lectin and the ferritin-avidin complexes. In control rough microsomes (*a*) lectin binding sites are normally found only on the luminal aspect of the membranes, whereas in inverted rough vesicles they are exposed on the convex face, which bears no ribosomes (*b*). The absence of lectin binding sites on the face opposite the ribosomes is apparent in inverted vesicles with visible discontinuities in their limiting membranes (*inset* in *b*). No ferritin binding was observed in controls from which Con A-biotin was omitted. Bar, 0.2 μm. × 120,000.

TABLE III

Puromycin-induced Release of Ribosomes from Control RM and from a Fraction Containing Inverted Vesicles

Sample	RNA in each, μg (%)		
	Supernatant and cushion fraction	Sedimentable fraction	Recovery of RNA
			%
1. RM	56 (12)	430 (88)	97
	73 (15)	405 (85)	97
2. RM + puromycin	395 (83)	79 (17)	94
	410 (82)	93 (18)	102
3. RM inverted	91 (19)	378 (77)	98
	63 (13)	421 (89)	94
4. RM inverted + puromycin	182 (39)	287 (61)	94
	142 (31)	321 (69)	93

Control RM and a fraction treated for inversion (*RM inverted*) (for details, see legend to Fig. 7) were resuspended in HSB at a final RNA concentration of 1 mg/ml. Aliquots (500 μl) of each sample received either 50 μl of water (samples *1* and *3*) or 50 μl of puromycin (34) (10^{-2} M; samples *2* and *4*). After incubation for 30 min at 0°C and 10 min at 37°C, all four samples were layered on top of 4 ml of a sucrose (10%) cushion containing HSB and centrifuged for 30 min at 60,000 *g*. Sedimentable fractions and the supernatants were separated and their RNA content was measured in cold TCA (10% final) precipitates. Recoveries were calculated as the sum of RNA found in the supernatant and pellet fractions and are expressed as percent of the RNA input (500 μg). The results of two independent experiments are presented.

ribophorins within the membrane at low concentrations may then be directly responsible for the change in configuration that is manifested in the formation of invaginations. Sheetz and Singer (36) have proposed an interesting "bilayer couple" hypothesis to explain shape changes induced by membrane perturbants. They have pointed out that, because proteins and lipids are asymmetrically distributed in the two halves of a membrane bilayer, each of these halves may contract or expand to a different extent when subjected to some perturbation. Because membranes form closed surfaces, invaginations would be produced when in a membrane vesicle the area of the inner half of the phospholipid bilayer expands or when the outer half contracts. An increment in the area of the inner half could be brought about by the selective intercalation of Triton X-100, whereas an accumulation of the asymmetrically inserted membrane ribophorins in the regions bearing ribosome aggregates could cause a contraction of the outer membrane. Because the accumulation of ribophorins that occurs in Kyro EOB–treated vesicles does not induce invaginations or affect the curvature of the membrane, we suggest that invaginations are caused by the selective incorporation of the Triton X-100 in the inner half of the bilayer.

The observation that ribophorins redistribute together with the ribosomes in the inverted vesicles produced by Triton X-100 suggests that these proteins are directly or indirectly connected to the ribosomes, as expected for components of the ribosome binding sites or for membrane elements that participate in the vectorial discharge or in the processing of nascent polypeptides. This was originally suggested by the fact that Kyro EOB, which causes the differential extraction of most proteins other than the ribophorins, preserves the association of ribosomes with a remnant that resembles the original rough microscome membrane (12–15).

The usefulness of the inverted rough microsomal vesicles in

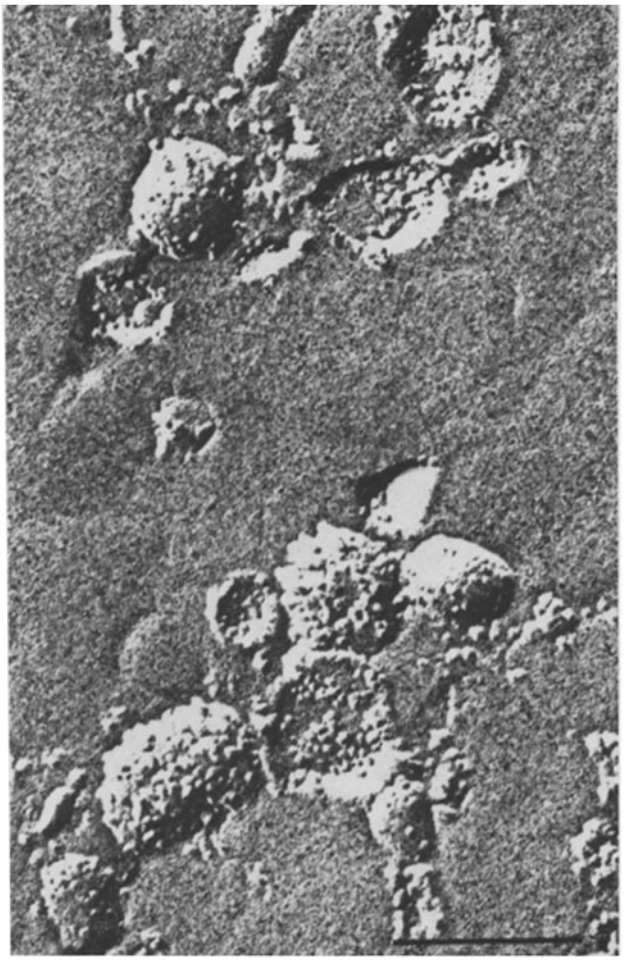

FIGURE 10 Freeze-fracture electron micrographs of inverted rough vesicles. Inverted rough vesicles (prepared as described in the legend to Fig. 7) were fixed with glutaraldehyde (1%), sedimented, impregnated with glycerol (30%), and processed for freeze-fracture. In contrast to the native rough microsomes (29), concave and convex fracture faces of inserted rough vesicles have comparable densities of intramembrane particles. Bar, 0.2 μm. × 125,000.

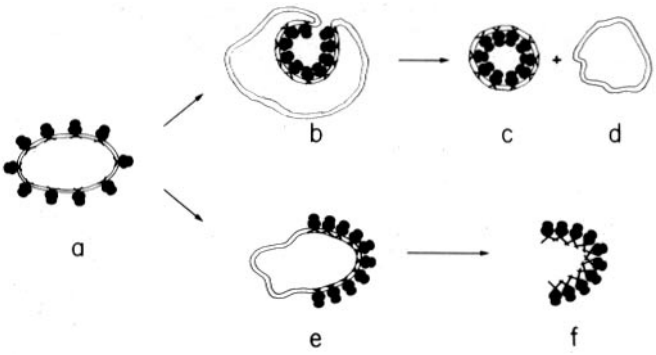

FIGURE 11 Schematic drawing depicting different stages observed during the subfraction of RM with nonionic detergents. Very low concentrations of Triton X-100 (0.08%) cause the formation of ribosome aggregates on regions of the membrane that invaginate towards the microsomal lumen (*b*). Slightly higher detergent concentrations (0.16%) lead to the fragmentation of rough microsomes into inverted rough vesicles (*c*) and smooth-surfaced vesicles (*d*). On the other hand, low concentrations of Kyro EOB (0.12%) lead mainly to the aggregation of ribosomes, which form caps on the convex surface of the microsomes (*e*). Higher detergent (0.5–1%) concentrations dissolve most proteins, leaving curved membrane remnants that have ribosomes bound to convex faces (*f*).

studies concerned with the transmembrane disposition of various microsomal membrane proteins and with the sidedness of functional activities associated with the apparatus for vectorial transfer and processing of nascent polypeptides will be examined in future work using impermeant labeling reagents and proteolytic probes.

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