

SELECTIVE RELEASE OF CONTENT FROM MICROSOMAL VESICLES WITHOUT MEMBRANE DISASSEMBLY

I. Permeability Changes Induced by Low Detergent Concentrations

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ABSTRACT

Rat liver rough microsomes treated with a series of desoxycholate (DOC) concentrations from 0.003 to 0.4% were analyzed by isopycnic sucrose density gradient centrifugation in media containing high or low salt concentrations. Tritium-labeled precursors administered *in vivo* were used as markers for ribosomes (orotic acid, 40 h), phospholipids (choline, 4 h), membrane proteins (leucine, 3 days), and completed secretory proteins of the vesicular cavity (leucine, 30 min). Within a narrow range of DOC concentrations (0.025–0.05%), the vesicular polypeptides were selectively released from the microsomes, while ribosomes, nascent polypeptides, and microsomal enzymes of the electron transport systems were unaffected. The detergent concentration which led to leakage of content was a function of the ionic strength and of the microsome concentration. At the lowest effective DOC concentration the microsomal membranes became reversibly permeable to macromoles as shown by changes in the density of the vesicles in Dextran gradients and by the extent of proteolysis by added proteases. Incubation of rough microsomes with proteases in the presence of 0.025% DOC also led to digestion of proteins from both faces of the microsomal membranes and to a lighter isopycnic density of the membrane vesicles.

INTRODUCTION

Membranes of the endoplasmic reticulum (ER) are complex structures of considerable physiological importance which serve as permeability barriers between two main subcellular compartments. One side of the ER membrane faces the cell sap, provides sites for the attachment of ribosomes manufacturing specific protein types, and accepts various substrates for synthesis, degradation, or biochemical modification (see review by Siekevitz, 1972). The opposite side of the membrane limits the cisternal space or lumen of the

ER, where products of membrane-bound ribosomes are transferred and segregated. Enzymatic systems on this side appear to be concerned with glycosidation (Schachter et al., 1970), hydroxylation (Estabrook et al., 1971), or cross-linking of secretory products by formation of disulfide bonds (DeLorenzo et al., 1966).

It is thought that during cell fractionation there is little opportunity for leakage of the cisternal content of the ER, and that this is preserved in the lumen of microsomes (Palade and

Siekevitz, 1956). Rough microsomes have been shown to act *in vitro* as functional units for the synthesis and transfer of polypeptides (Redman et al., 1966; Redman and Sabatini, 1966; Redman, 1967, 1969) and to be capable of some steps in the modifying sequence affecting secretory products (Redman and Cherian, 1972). While the complex of ribosomes and membrane vesicles in a rough microsome has recently been functionally disassembled *in vitro* (Adelman et al., 1973b), a nondestructive and effective procedure for the separation of the content of microsomes from the limiting membranes is still required to elucidate questions concerning the fate and the processing of products from membrane-bound ribosomes. Osmotic shock and mechanical disruption by sonication, Ultra Turrax, or French press treatment (Campbell et al., 1960; Dallner, 1963; Redman, 1969; Redman and Cherian, 1972) have been extensively used to produce leakage of content from closed vesicles. However, release of secretory proteins by osmotic shock is inefficient, and mechanical disruption, if effective, leads to deleterious effects on ribosomes (Dallman et al., 1969) and membranes (Tsukagoshi and Fox, 1971). Ernster et al., (1962) first attempted to separate membranes from the luminal content by controlling the effect of detergents on membrane disassembly. This procedure, using 0.25% dexoxycholate (DOC) on concentrated microsome samples, has been used frequently (Redman et al., 1966; Redman and Sabatini, 1966; Redman, 1967; Redman and Cherian, 1972), although it leads to partial solubilization of phospholipids and microsomal enzymes and to extensive ribosome detachment (Ernster et al., 1962). In this paper we present evidence indicating that at critical ratios of detergent to microsomal concentrations, structural changes can be produced in microsomal membranes which allow the leakage of content from rough microsomes without leading to a loss of bound ribosomes or to extensive membrane disassembly.

MATERIALS AND METHODS

General

Solutions, prepared using deionized distilled water, were passed through Millipore filters (0.45 or 1.2 μm for concentrated sucrose solutions) and stored in the cold. Centrifugations were carried out in an IEC B-60 centrifuge. A notation such as 15 min-

40K-A321 is used to denote a 15-min centrifugation at 40,000 rpm in the A321 rotor (Adelman et al., 1973a). All pH's were measured at room temperature.

Materials

Enzyme grade sucrose was obtained from Schwarz/Mann Research Laboratories, N. Y.; Tris-base (Trizma), from Sigma Chemical Co., St. Louis, Mo.; bovine serum albumin (3 \times recrystallized) and sodium desoxycholate (NaDOC) from Armour Pharmaceutical Co., Chicago, Ill.; 3 \times crystallized trypsin and α -chymotrypsin from Worthington Biochemical Corp., Freehold, N. J.; Ficoll and Dextran T40 from Pharmacia, Piscataway, N. J.; Puro-mycin dihydrochloride from Nutritional Biochemical Corp., Cleveland, Ohio; NCS from Amersham/Searle, Arlington Heights, Ill.; Liquifluor from New England Nuclear Corp., Boston, Mass.; Triton X-100 from Rohm and Haas; and desoxycholic acid (carboxyl- ^{14}C , specific activity 41.2 mCi/mmol) from ICN, Irvine, Calif.

Solutions

TKM is 50 mM Tris-HCl pH 7.5, 25 mM KCl and 5 mM MgCl_2 ; 0.25 M STKM is TKM with 0.25 M sucrose; similarly, 2.0 STKM is TKM with 2.0 M sucrose. Low salt buffer (LSB) refers to 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 5 mM MgCl_2 , modifications are mentioned in the text. High salt buffer (HSB) refers to 50 mM Tris-HCl, pH 7.5, 500 mM KCl, and 5 mM MgCl_2 . 5% and 7.84% (2×10^{-1} M) stock solutions of DOC were prepared by dissolving NaDOC and adjusting the pH to 7.5 at 20°C with 0.1 N HCl.

Fractionation of Liver Cells

Male albino rats of the Sprague-Dawley strain (120–150 g) were maintained on a Purina Chow diet and fasted for 20 h before sacrifice with a guillotine (Harvard Apparatus Co., Inc., Dover, Mass.).

Rough microsomes (RM) and smooth microsomes (SM) were prepared by the procedure of Adelman et al. (1973a). Microsome pellets (10–30 mg protein per fraction) were stored at -20°C for up to 2 mo. Alternatively, and to reduce aggregation (Borgese et al., 1973), microsomes were resuspended in 0.25 M STKM, mixed with 2 vol of glycerol, and kept at -20°C . Before use, the microsomes stored in glycerol were diluted four times with a solution containing 0.25 M sucrose, 500 mM KCl, 50 mM Tris-HCl, and 10 mM MgCl_2 , and were recovered by sedimentation (15 min-40K-A321). Microsomes stored as pellets were also washed once in 0.25 M sucrose HSB before using.

From 22 g rat liver (four to five rats), an average of 180 mg protein was recovered in RM and 190 mg protein in SM. The puromycin-KCl procedure (Adelman et al., 1973b) was used to prepare microsomal membranes stripped of ribosomes. Suspensions of RM in HSB (3–6 mg protein per ml) were incubated with puromycin ($\sim 1 \times 10^{-3}$ M) for 30 min at room temperature and for 10 min at 37°C. Stripped membranes were recovered by sedimentation (20 min-30K-A211) after 10 × dilution with HSB.

Density Gradient Analysis

Linear sucrose density gradients (~ 12 ml) were prepared in nitrocellulose tubes (Beckman Instruments, Palo Alto, Calif.) by standard techniques (Britten and Roberts, 1960). Centrifugation was at 3°C, and the centrifuge was stopped without braking. The gradients were withdrawn from the top, and the optical density at 254 nm was monitored as previously described by an LKB Uvicord (Adelman et al., 1973b). In all figures the direction of sedimentation is from left to right. When necessary, fractions of about 0.5 ml were collected from the pump effluent for determination of protein and radioactivity throughout the gradient. Pellets were resuspended in 0.5 ml TKM for chemical and radioactivity measurements.

Linear Ficoll (10–40% wt/vol) and Dextran T-40 (10–30% wt/vol) density gradients (in modified HSB containing 2 mM MgCl₂) were prepared with a gradient mixer of conical chambers and a vibrating stirring device (Buchler Instruments, Fort Lee, N. J.). Due to the high viscosity the effluent of the mixer was led into the tubes by a peristaltic pump (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.). Gradient fractions of 0.5 ml were collected using graduated conical tubes. Aliquots were used for radioactivity determinations and measurements of optical density at 260 and 280 nm after 1:1 dilution with water.

Chemical and Radioactivity Determinations

Protein was determined in duplicate aliquots according to Lowry et al. (1951), using bovine serum albumin (BSA) as a standard. The concentration of BSA was determined from the OD at 279 nm (Foster et al., 1965).

The *RNA* content of microsomes was estimated from the OD₂₆₀ of samples treated with 0.5% DOC (ribosomes $E_{260}^{1\%} = 135$; Tashiro and Siekevitz, 1965).

Phospholipid (PL) extracts were prepared essentially according to Folch et al. (1957). Aliquots of the organic phase were taken, the solvent was evaporated, and phosphorus was measured according

to Ames and Dubin, (1960). Inorganic phosphate was used as a standard, and it was assumed that 25 mg phospholipid contains 1 mg phosphorus.

Total *radioactivity* was measured without TCA precipitation in aliquots (10–200 μ l) which received NCS and were counted with 8 ml Toluene-Liquifluor as scintillator.

To determine [³H]- and [¹⁴C]leucine radioactivity incorporated in TCA-insoluble material, 100- μ l aliquots were pipetted onto Whatman 3 MM filter paper discs (diameter 2.3 mm), which were processed as described by Mans and Novelli (1961). To measure radioactivity in RNA and the incorporation of [³H]D-glucosamine, the heating to 90°C in 5% TCA was omitted. Filters were counted with 5 ml of Toluene-Liquifluor as scintillation fluid in a Beckman Model LS 250, or in a Mark I Nuclear Chicago scintillation counter.

In Vivo Labeling and Distribution of Radioactivity in Cell Fractions

Table I lists the radioactive labeled precursors injected into rats, the specific radioactivity of each, and the time of in vivo labeling elapsed before sacrifice. Volumes of solutions injected varied from 0.2 to 0.5 ml. When radioactive leucine was administered, different time periods of in vivo labeling were chosen to obtain incorporation of this amino acid into nascent chains (2 min) or into completed but recently synthesized proteins (30 min). Proteins which are stable and remain in the microsomes a minimum of 1 day, were labeled by injecting the precursor once every day for 3 days and waiting for 24 h after the last injection before sacrifice. For the 2 min labeling with [³H]- or [¹⁴C]leucine, the amino acid was injected into the portal vein under ether anesthesia; other injections were given intraperitoneally. For convenience, proteins labeled for 2 min will be called “pulse-labeled” proteins, whereas proteins labeled for 30 min before sacrifice will be called “short-term labeled” proteins, in contrast to stable or “long-term labeled” proteins.

Table II shows the distribution of radioactivity in rat liver cell fractions and in serum obtained after 2 min, 30 min, or long-term administrations of radioactive leucine (1–3 days). The distributions are summarily described here to justify the use of these preparations for the experiments described in this and a following paper.

The amount of labeled polypeptides in free polyosomes which are recovered as a pellet corresponds to the difference between the radioactivity in the postmitochondrial supernatant (PMS) and the sum of the radioactivities in smooth microsomes (SM), rough microsomes (RM), and final supernatant. This difference was most significant 2 min after the

TABLE I

In Vivo Incorporation of Radioactively Labeled Precursors into Rough and Smooth Microsomes from Rat Liver

Preparation	Radioactively labeled precursor	Specific activity	$\mu\text{Ci/g}$ rat injected*	Sacrificed after	SM ⁺		RM ⁺	
					Specific activity	% of injected radioactivity	Specific activity	% of injected radioactivity
a	Choline-[methyl- ³ H]chloride‡	0.55	1.6	4 h	—	—	5.2×10^4 §	0.48
b	[5- ³ H]Orotic acid‡	2.81	1	40 h	—	—	5.0×10^4	0.8
c	L-[¹⁴ C]leucine	0.312	3×0.83 ¶	24 h	1.9×10^4	0.17	2.5×10^4	0.23
d ₁	L-[4,5- ³ H]leucine	58.0	1	30 min	6.4×10^4	0.8	4.8×10^4	0.6
d ₂	L-[4,5- ³ H]leucine	58.0	1	30 min	7.3×10^4	0.9	6.6×10^4	0.8
d ₃	L-[4,5- ³ H]leucine	40.0	3.2	30 min	1.6×10^5	0.7	1.5×10^5	0.63
e ₁	L-[¹⁴ C]leucine**	0.344	0.1	2 min	—	—	7.5×10^3	1.2
e ₂	L-[4,5- ³ H]leucine	40.0	4.0	2 min	2.8×10^5	2.3	5.1×10^5	3.0
e ₃	L-[4,5- ³ H]leucine	40.0	1.0	2 min	6.1×10^4	1.1	9.6×10^4	1.4
f	D-[³ H]Glucosamine GL‡‡	1.9	3.5	30 min	8.4×10^4	0.39	6.0×10^3	0.024

+Recovery of protein in RM and SM was 8.2 mg and 8.7 mg, respectively, per gm of liver.

* In preparation a-d₃ and f the isotopes are injected i.p. In preparations e₁-e₃ the isotopes are injected into the portal vein.

‡ New England Nuclear Corp., Boston, Mass.

§ After Folch extraction (Folch et al., 1957) 92% of the radioactivity in the RM was found in the chloroform phase.

|| Schwarz BioResearch, Orangeburg, N. Y.

¶ (Long-term labeling). The rat received three injections at 24-h intervals. 24 h after the last injection the animal was sacrificed.

** Amersham/Searle, Des Plaines, Ill.

‡‡ International Chemical and Nuclear Corp., Irvine, Calif.

administration of labeled leucine, at which time ribosomes contained highly labeled nascent chains. Although there were high levels of unincorporated leucine in the blood (3×10^6 dpm/ml serum), labeled plasma proteins had not yet been secreted at 2 min. Therefore, all labeled completed proteins (secretory or not) were still intracellular (see also Peters et al., 1971). Approximately half of the radioactivity in the 2 min labeled RM was not ribosome bound since it was released in completed chains when the sample was treated with 0.5% DOC.

The radioactivity found in the final supernatant before secretion into the blood occurs (i.e., 2 min) amounts to ~20% of the total acid-insoluble radioactivity. If this fraction is attributed to chains completed and released from free polysomes (which represent ~20% of the total ribosome preparation [Adelman et al., 1973a]), and the rates of synthesis by free and bound ribosomes are assumed to be approximately equal, it can be concluded that most of the completed proteins manufactured by bound

ribosomes were retained in the microsomes after cell fractionation.¹

After 30 min a large fraction of labeled plasma proteins has been secreted into the blood (serum radioactivity, Table II). Most labeled polypeptide chains have also been discharged from ribosomes. Correspondingly, a higher percentage of labeled proteins was found in the cell sap, and the microsomal radioactivity (RM and SM), which was only ~27% of the total, was completely DOC soluble. All these changes, as well as the fact that the total intracellular radioactivity, as a percentage of the applied isotope, was lower at 30 min than at 2 min

¹ Although at 2 min rough microsomes and smooth microsomes have similar percentages of the total incorporated leucine, the specific activity in secretory proteins is higher in rough microsomes, since there is a higher concentration of secretory proteins, such as albumin in smooth microsomes (Peters et al., 1971).

TABLE II
Distribution of Acid-Insoluble Radioactivity in Rat Liver Cell Fractions*

	2 min (pulse) (preparation e) [‡]	30 min (short term) (preparation d) [‡]	3 days (long term) (preparation c) [‡]
Total homogenate [§]	100% (3.34×10^8 dpm)	100% (3.34×10^8 dpm)	100% (2.1×10^7 dpm)
% of injected isotope recovered in homogenate	17.8%	5.0%	3.2%
Postnuclear supernatant	92.1%	84%	81%
Postmitochondrial supernatant	78.2%	73%	67%
Final supernatant	21.2%	45%	53%
Smooth microsomes	20.8%	14%	4.8%
Rough microsomes	26.6%	13%	6.6%
TCA-precipitable radioactivity in serum, dpm/ml	0.0%	3.21×10^6	2.4×10^6

* See Table I for labeling conditions.

[‡] Preparations are those listed in Table I.

[§] TCA-precipitable dpm in ~25 g of liver.

(Table II), reflect the protein export which is known to proceed in this interval (see also Peters et al., 1971; Glaumann, 1970; Glaumann and Ericsson, 1970).

The distribution of radioactivity in the fractions obtained after long-term labeling showed that most of the intracellular label was incorporated into completed protein found in the cell sap (~53%) or into sedimentable cellular structures. Ribosomes contained negligible radioactivity, and in the microsomes, only ~11% of the total radioactivity was recovered.

In serum from rats labeled for 30 min and by the long-term treatment, practically all radioactivity was acid insoluble (Table II).

Cytochrome b_5 and NADH and NADPH Cytochrome c Reductases

Cytochrome b_5 concentration was estimated from the difference between the maximum (424 nm) and minimum (409 nm) absorbance in the difference spectrum (reduced minus oxidized cytochrome b_5) measured in a Cary-14 recording spectrophotometer at room temperature (Garfinkel, 1957). Reduction was achieved by adding 20 μ l of a 100 mM $\text{Na}_2\text{S}_2\text{O}_4$ solution dissolved in 100 mM NaOH (kept under nitrogen) to one of the cuvettes (1-cm light path). NADH and NADPH cytochrome c reductases were measured at 25°C in microsomes added to a system containing 50 mM Tris-HCl buffer pH 7.5, 0.1 mM NADH or NADPH, 0.05 mM cytochrome c and 0.33 mM KCN in a final volume of 1 ml. The reduction of cytochrome c was followed at 550 nm in a Gilford Model 240 recording spectrophotometer with an automatic cuvette changer (Gilford Instru-

ments Laboratories, Inc., Oberlin, Ohio). Only the linear part of the curve was used to calculate the enzymatic activity, which is expressed as nanomoles of cytochrome c reduced per minute per milligram protein. A value of 18.5×10^3 liter/mole per cm was used for the extinction coefficient of reduced minus oxidized cytochrome c at 550 nm (Omura et al., 1967).

Detergent Treatment

RM labeled in different constituents (see Table I) were washed in HSB (20 min-20K-SB283) and resuspended in LSB or HSB, using a Potter-Elvehjem homogenizer operated by hand. RM suspensions kept in the cold received DOC solutions of 10X the desired final detergent concentration. During addition of detergent, the suspension was mixed in a Vortex tube mixer. Samples were usually kept at 0°C for 30 min before loading onto gradients.

Electron Microscopy

Microsomes which were incubated with or without DOC and/or proteolytic enzymes in low or high salt buffers were fixed by addition of an equal volume of 4% glutaraldehyde maintaining the concentrations of detergent and the salt composition but with triethanolamine (TEA instead of Tris.) Microsomes were kept in fixative for 20 min and sedimented (30 min-39K-SW39). Alternatively they were first sedimented and fixed (60 min at 4°C) as pellets with a glutaraldehyde solution of the same salt composition. Samples were washed, postfixed with OsO_4 , stained in block with uranyl acetate, and embedded in Epon. Sections were stained with lead hydroxide.

RESULTS

a. Effect of DOC on Microsomal Membranes and Release of Microsomal Content

The effect of increasing concentrations of DOC on the microsomal vesicles was assessed by sucrose density gradient analysis from changes in the sedimentation behavior of different microsomal constituents labeled *in vivo* (Table I). The distribution of phospholipid, RNA, protein, or glycoprotein was determined after zone sedimentation in sucrose gradients (10–50% SHSB or 10–60% SLSB). Conditions of centrifugation (3 h-40K-SB283) were sufficient to sediment microsomal membranes (peak labeled Mb in Fig. 1) to an isopycnic position. Depending on the previous treatment, the density of the vesicles varied within the range 1.16–1.21.

Comparison of the absorbance and radioactivity profiles obtained from detergent-treated RM containing labeled phospholipids (^3H]-choline-4 h, preparation a, Table I) indicated that concentrations of DOC lower than 0.098% had no detectable effect on the sedimentation properties of the membranes or on the distribution of labeled phospholipids. As can be observed in Fig. 1, ribosomes were not released from the microsomal vesicles (peak labeled Mb) until membrane breakdown occurred at 0.098% DOC. The joint appearance of radioactive ^3H]-choline in the top part and the absorbance profiles of ribosomes in the middle part of the gradients indicates membrane breakdown (Fig. 1 d, e, f). Total solubilization of the RM membranes was only observed after treatment with 0.392% DOC (Fig. 1 f). At this DOC concentration most of the labeled phospholipids remained within the sample zone, and typical polysome patterns appeared within the gradient.

The stability of the microsomal membranes to increasing DOC concentrations was also tested using microsomes containing long-term labeled proteins, i.e., proteins labeled after 3 daily injections of ^{14}C]-leucine, the last one given 1 day before sacrifice (preparation c, Table I). This schedule was chosen to label mainly structural components of the microsomes, since secretory proteins are known to be rapidly discharged into the bloodstream after synthesis in the ER. Analysis of the samples (not shown) demonstrated that throughout the range of DOC concentrations most of the long-term labeled microsomal

protein behaved similarly to the ^3H]-choline-labeled phospholipids in Fig. 1. As expected, however, a small fraction of the radioactivity in long-term labeled proteins was incorporated into ribosomes released at DOC concentrations higher than 0.098%.

Strikingly different results were obtained when microsomal proteins were labeled with ^3H]-leucine administered only 30 min before sacrifice

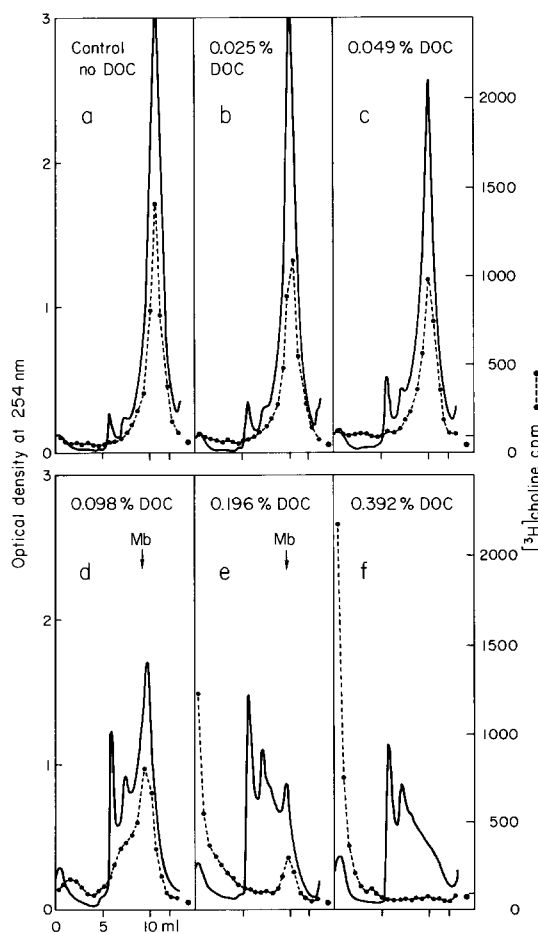


FIGURE 1 Effect of increasing concentrations of DOC on the integrity of microsomal membranes. RM containing labeled phospholipids (^3H]-choline-4 h, preparation a, Table I) were resuspended (3–4 mg protein per ml) in LSB. Aliquots (500 μl) which were kept at 0°C for 30 min, with different concentrations of DOC, were loaded onto linear sucrose density gradients (10–60% SLSB). After centrifugation (3 h-40K-SB283) at 3°C, the absorbance at 254 nm was recorded and the radioactivity measured in 100- μl aliquots taken from 0.5 ml fractions. —, OD_{254} , ●—● ^3H]-choline radioactivity.

(short-term labeled proteins: [^3H]leucine-30 min, preparation d_2 , Table I). It is known that after this time of in vivo incorporation, liver secretory proteins, which are manufactured in the ER, are extensively labeled, although nascent polypeptide chains, growing in bound ribosomes, are no longer significantly radioactive (Peters et al., 1971; Glaumann and Ericsson, 1970; Glaumann, 1970; Table II). As the profiles in Fig. 2 show, after treatment with DOC concentrations as low as 0.025% and 0.049%, large fractions (42% and 56%, respectively) of the total radioactivity were released from the vesicles and no longer sedimented with the membrane band. On the other hand, control RM (washed with HSB), which were incu-

bated at 4°C for ~30 min before gradient centrifugation but were not treated with detergent, released only ~10% of the short-term labeled protein.

Fig. 3 summarizes data on the detergent-dependent release of several labeled components from rough microsomes. For each marker and for each DOC concentration the fraction of label associated with the membrane band or found in the upper regions of the gradient was computed from analyses similar to those illustrated in Figs. 1 and 2. Two families of curves were recognized corresponding to two different release behaviors of microsomal constituents. Structural components of microsomes such as phospholipids ([^3H]choline-4

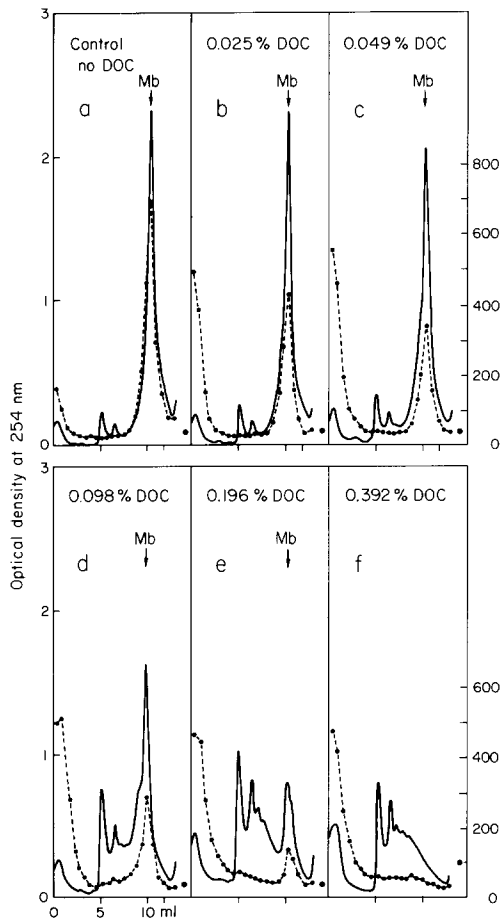


FIGURE 2 Effect of DOC on the release of short-term labeled proteins from rough microsomes. RM ([^3H]leucine-30 min, preparation d_2 , Table I) were treated and analyzed as described in the legend for Fig. 1. —, OD_{254} , ● — ●, [^3H]leucine radioactivity.

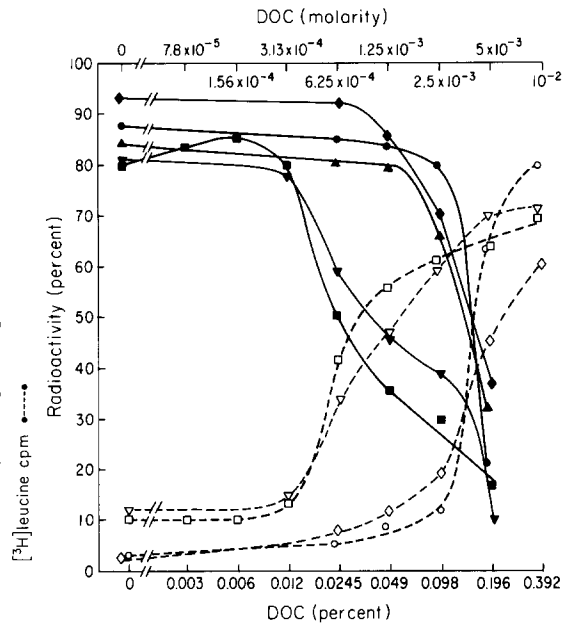


FIGURE 3 Differential effect of DOC on the release of labeled microsomal constituents in a solution of low ionic strength (LSB). RM containing labeled phospholipids ([^3H]choline-4 h, preparation a, Table I); ribosomes ([^3H]RNA-[^3H]orotic acid-40 h, preparation b, Table I); glycoproteins ([^3H]glucosamine, preparation f, Table I); proteins labeled in vivo for 30 min ([^3H]leucine-30 min, preparations d_1 and d_2 , Table I) or for 1-3 days ([^{14}C]leucine long-term, preparation c, Table I) were resuspended in LSB, treated, and analyzed as described in Fig. 1. — — — Percent of total radioactivity in first five fractions near the top of the gradient; — — — percent of total radioactivity in seven fractions corresponding to the membrane band; ○ ● 4 h [^3H]choline; ▲ 40 h [^3H]orotic acid; ■ □ 30 min [^3H]leucine; ◇ ◆ long-term [^{14}C]leucine; ▽ ▼ 30 min [^3H]glucosamine.

h, preparation a, Table I), proteins of slow turnover (^{14}C]leucine long-term, preparation c, Table I), and ribosomes (^3H]orotic acid-40 h, preparation b, Table I) were not appreciably released until microsomes were treated with a DOC concentration of 0.196% or higher. These behaved in a strictly parallel fashion throughout the whole range of DOC concentrations tested. On the other hand, short-term labeled proteins (^3H]leucine-30 min) and glycoproteins (^3H]glucosamine-30 min, preparation f, Table I) were already significantly released at DOC concentrations as low as 0.025% and 0.049%. The DOC concentration at which the crossover of curves for membrane-associated and "soluble" radioactivity occurs in Fig. 3 was taken as a measure of the stability of the association of each constituent with the microsomal vesicle. It is clear that there was a fourfold concentration difference in the levels of DOC required to release short-term and long-term leucine-labeled proteins.

It should be noted, however, that the behavior of ^3H]glucosamine-labeled glycoproteins was not strictly parallel to the behavior of the short-term ^3H]leucine-labeled proteins. The association of the former with the sedimentable membranes was more stable at higher DOC concentrations. This suggests that at least part of the ^3H]glucosamine label was incorporated into membranes or into microsomal components more tightly associated with the membranes than the bulk of the short-term labeled proteins, most of which can be presumed to be secretory.

b. Effect of Ionic Strength on the Stability of Microsomes to DOC Treatment

The DOC concentration which first induced a selective release of short-term labeled proteins and glycoproteins from microsomes depended on the ionic strength of the medium of incubation. Fig. 4 illustrates the release of short-term labeled proteins (^3H]leucine-30 min) from microsomes brought to a medium of high ionic strength (HSB), incubated with a series of DOC concentrations, and analyzed in gradients of the same ionic composition. Although the leakage of labeled protein observed in control microsomes in HSB (~10%) was not increased with respect to the value in LSB, comparison with Fig. 2 shows that in the higher salt medium (0.5 M KCl) a lower DOC concentration was sufficient to induce the selective release

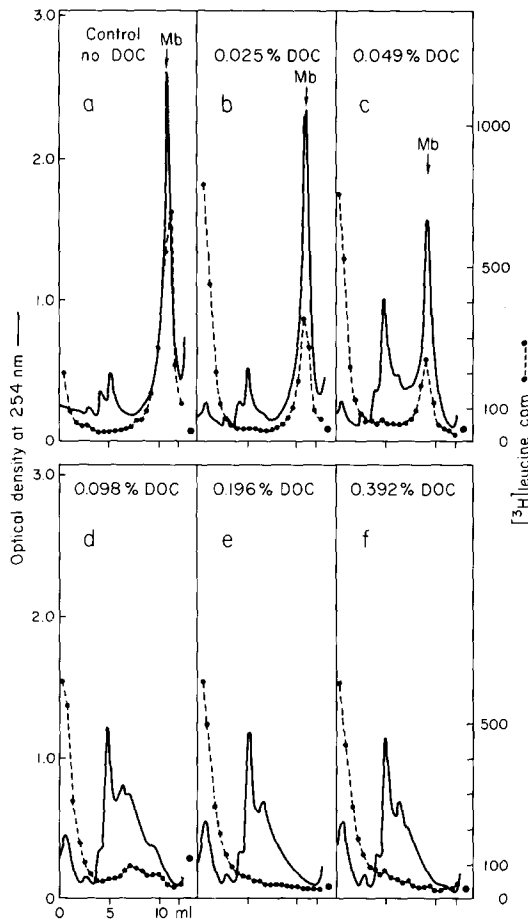


FIGURE 4 DOC-dependent release of short-term labeled proteins from RM in a solution of high ionic strength. RM (^3H]leucine-30 min, preparation d₂, Table I) were resuspended in LSB and brought to HSB by addition of a compensating solution. Samples were incubated with DOC, loaded onto linear sucrose density gradients (10–50% SHSB), centrifuged, and analyzed as described in the legend for Fig. 1.

of short-term labeled proteins. In HSB a release as extensive as 54% of the total radioactivity was already observed at 0.025%, and at ~0.049% DOC, ribosome release was already apparent (optical density profile in Fig. 4). The results of a series of analyses in HSB with differently labeled microsomes are summarized in Fig. 5, which is to be compared with Fig. 3. Although the behavior of easily releasable proteins (^3H]leucine-30 min) and glycoproteins (^3H]glucosamine-30 min) versus tightly associated components such as phospholipids (^3H]choline-4 h) and ribosomes (^3H]orotic acid-40 h) is still observed, both occur at

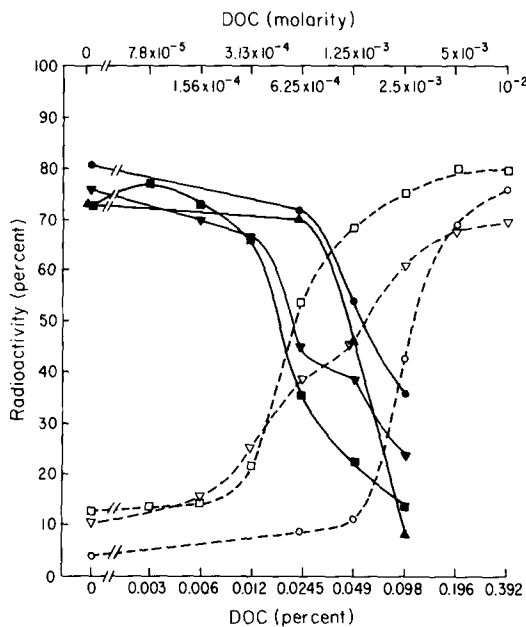


FIGURE 5 Differential effect of DOC on the release of labeled microsomal constituents in a solution of high ionic strength (HSB). RM (3–4 mg protein per ml) in HSB, labeled as indicated in the legend for Fig. 3, were treated with DOC and loaded onto gradients (10–50% in SHSB) which were centrifuged and analyzed as described in the legend to Fig. 1. — — — Percent of total radioactivity in first five fractions near the top of the gradient; — — — percent of total radioactivity in seven fractions corresponding to the membrane band; ○ ● 4 h $[^3\text{H}]$ choline; ▲ 40 h $[^3\text{H}]$ orotic acid; □ ■ 30 min $[^3\text{H}]$ leucine; ▽ ▼ 30 min $[^3\text{H}]$ glucosamine.

approximately half the concentration value needed to effect the release in LSB. The difference in release behavior between glycoproteins and short-term labeled proteins observed in LSB was reproducibly more pronounced in HSB. As can be seen in Fig. 5, glycoproteins show a biphasic curve of release; at low DOC concentration ($\sim 0.025\%$), as much as 35% of the $[^3\text{H}]$ glucosamine label was released in parallel with the release of short-term labeled protein; but at DOC concentrations higher than $\sim 0.049\%$, the curve of release of $[^3\text{H}]$ glucosamine label approached the curve of disassembly of membranes which is represented by the appearance of the tightly associated phospholipids ($[^3\text{H}]$ choline-4 h) in the supernatant.

c. Effect of the Ratio of Detergent to Microsome Concentration

Numerous previous reports (e.g., Ernster et al., 1962; Engelman et al., 1967; Philippot, 1971; Small, 1971) suggest that the ratio of detergent to the amount of phospholipid present in membranes determines the level of release of membrane proteins. We studied this phenomenon in rough microsomes, following the dependency of the DOC effect on membrane concentration at two fixed levels of DOC; 0.049% and 0.196% (5 $\times 10^{-3}$ M) DOC (Fig. 6), a decrease in microsomal concentration below 25 mg protein per ml caused membrane dissolution, as indicated by the height of the absorbancy peak at the membrane region and by the release of ribosomes. On the other hand, short term-labeled proteins ($[^3\text{H}]$ leucine-30 min) were largely released at a microsome concentration (25 mg protein per ml) at which membrane integrity appeared unaffected. Thus, the complete sequence of release, first of short-term labeled proteins and then of membrane constituents, proceeded at a single DOC concentration. On the other hand, when the DOC concentration was 0.049% (not shown) and the RM concentration was varied from 25 mg protein per ml to 1.6 mg protein per ml, tightly associated membrane components were unaffected, judging, for example, from ribosome release and from the position and adsorbance of the membrane peak. However, the release of short-term ($[^3\text{H}]$ leucine-30 min) labeled proteins gradually increased with the decrease in microsome concentration. A degree of release similar to that obtained with 0.196% DOC and 25 mg/ml of microsomal protein was obtained at 0.049% DOC with 6.25 mg/ml of microsomal protein. Therefore the effective molar ratio of DOC to phospholipid was the same in both cases.

A quantitative summary of the effect of changing the ratio of DOC to RM (at fixed DOC concentration) is presented in Fig. 7, which shows that at the low (0.049%) DOC concentration the effect on the release of secretory proteins is linearly and inversely related to the protein concentration. On the other hand, the effect of membrane disassembly is only observed at 0.196% DOC and is represented by the anomalous bend in the curves of protein solubilized from the membranes which occurs at the RM concentration of ~ 6.25 mg protein per ml.

In order to determine to what extent DOC is

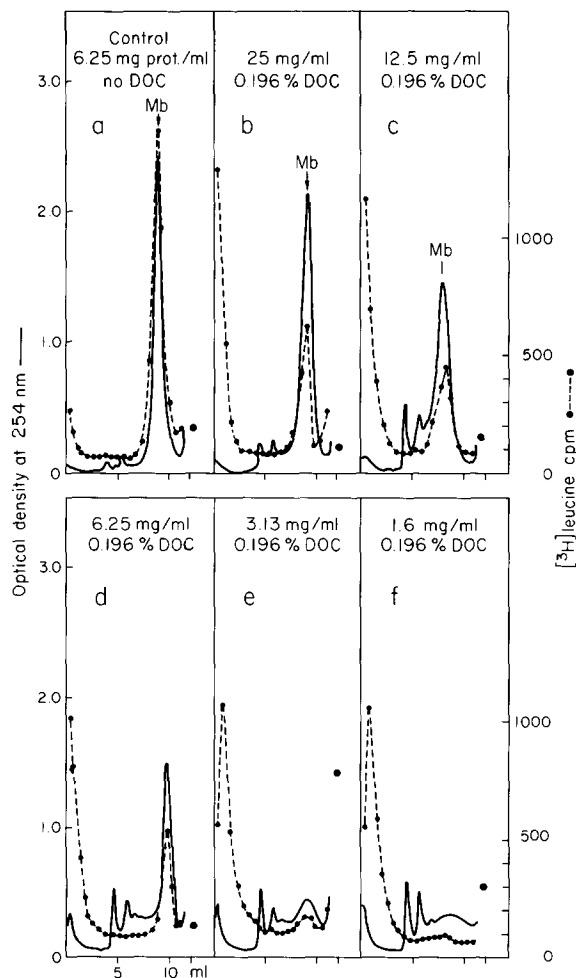


FIGURE 6 Effect of the ratio of detergent to microsome concentration. RM containing short-term labeled proteins (^3H leucine-30 min, preparation d_3 , Table I) were resuspended in LSB and adjusted to concentrations ranging from 25 mg protein per ml to 1.6 mg protein per ml. All samples except a control (6.25 mg protein per ml) received DOC to a final concentration of 0.196%. After 30 min at 0°C , 50 to 800- μl aliquots were loaded on top of sucrose gradients, all of which received approximately equal amounts of microsomes. Composition of the gradients, centrifugation, and analysis were as described in the legend to Fig. 1.

bound to microsomal membranes before it induces leakage or membrane disassembly, we followed the binding of radioactive ^{14}C -labeled DOC to RM membranes at levels of DOC which were either insufficient to produce leakage (0.0002%) or which produced leakage but did not dissolve the membranes (0.049%). The specific radioactivity of the DOC was adjusted so that the same amount of radioactivity was added at different DOC concentrations to a fixed amount of RM. Sucrose density gradient analysis showed (Fig. 8)

that an increase in DOC concentration of ~ 250 times (from Fig. 8 a to Fig. 8 b) caused a 100-fold increase in the total amount of DOC bound to the microsomal membranes (although only $\sim 2\%$ of the radioactivity is bound in Fig. 8 b vs. $\sim 5\%$ in Fig. 8 a). Taking the phospholipid to protein ratio of RM to be $\sim 0.330 \mu\text{mol}$, P/mg protein (Colbeau, et al., 1971, and our own measurements) the molar ratio of DOC to PL in the membrane band of gradients a, b in Fig. 8 is 2.5×10^{-4} and 2.5×10^{-2} , respectively. The results

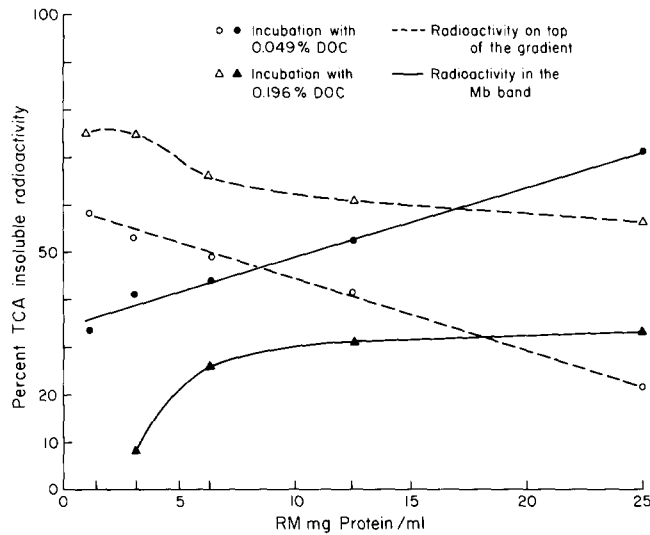


FIGURE 7 Summary of the effect of changing the ratio of DOC to rough microsomes at two fixed detergent concentrations. Data for 0.196% in LSB were computed from the results in Fig. 6, as described in the legend for Fig. 3. Data for 0.049% DOC in LSB were obtained from results of a similar experiment (not shown).

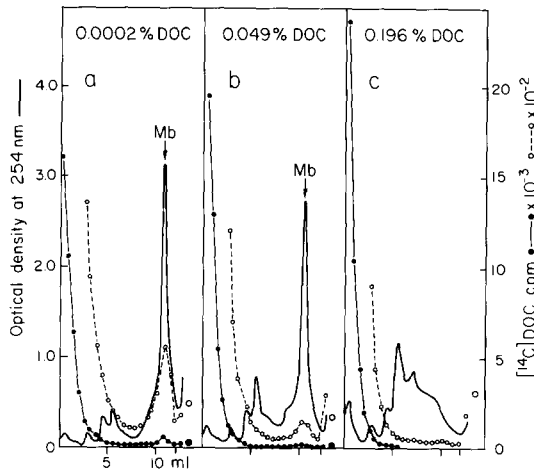


FIGURE 8 Binding of labeled DOC to rough microsomes. 0.5 ml aliquots of RM (3-4 mg protein per ml) in HSB were incubated for 30 min with the same amount of radioactive [^{14}C]DOC (2.5×10^5 dpm) and supplemented to a final DOC concentration of 0.0002% (a), 0.049% (b), and 0.196% (c). After 30 min at 0°C samples were loaded onto sucrose gradients (10-50% SHSB) which were centrifuged and analyzed as described in the legend to Fig. 1.

demonstrate that this amount of DOC can be bound by microsomal membranes before lysis occurs. In a following section (see Results, section g) it will be shown that a higher level of DOC is

also needed to maintain permeability changes which allow the passage of macromolecules through the membranes.

d. Stability of the Association of Enzymes with Microsomal Membranes

To be able to operationally define membrane proteins in this system and to distinguish them from proteins in the vesicular cavity, we examined the detergent-dependent release of enzymes which are presumed to be tightly bound to microsomal membranes, such as NADPH and NADH cytochrome *c* reductases and of cytochrome *b₅* (Omura et al., 1967). RM membranes treated with different DOC concentrations in LSB and components solubilized from them were separated by differential centrifugation. The activity of the enzymes and the cytochrome *b₅* concentration were determined in each fraction. Fig. 9 compares the release of each with the release of total protein and of short-term labeled proteins ($[^3\text{H}]$ leucine-30 min, preparation d_3 , Table I). It is clear that both cytochrome *c* reductases and cytochrome *b₅* behave as structural components of the membranes, and are more tightly bound to the vesicles than short-term labeled proteins. As expected (Fig. 9), the curve of total protein released was intermediary between those of secretory and structural components. Since at all concentrations of DOC

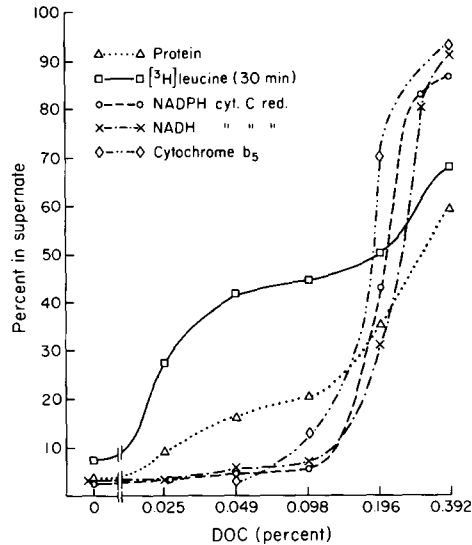


FIGURE 9 Stability of the association of NADPH and NADH-cytochrome *c* reductases with microsomal membranes. RM (³H]leucine-30 min, preparation d₃, Table I) were resuspended in a modified LSB (10 mM Tris-HCl, pH 7.5) to a final concentration of 3–4 mg protein per ml. 1- to 2-ml aliquots were incubated at 0°C for 30 min with DOC concentrations ranging from 0.025 to 0.392%. Sedimentable membranes and ribosomes were separated from the supernatant by differential centrifugation (60K-50 min-A269). Protein, TCA insoluble radioactivity, NADPH and NADH cytochrome *c* reductase activities (three experiments), and cytochrome b₅ concentrations (two experiments) were determined before fractionation as well as in the supernatant and in the resuspended pellet. For each sample, the 100% value was taken as the sum of the values in supernatant and pellet. In nonfractionated untreated RM, the specific activity of NADH and NADPH cytochrome *c* reductases was within 330–410 and 50–74 enzyme units per mg protein, respectively. The recovery of NADPH cytochrome *c* reductase (pellet plus supernatant) after treatment with DOC concentrations lower than 0.294% was consistently higher than 78%. For NADH cytochrome *c* reductase at DOC concentrations lower than 0.196%, the recoveries were better than 60%. At higher DOC concentrations recoveries of both enzyme activities were more variable.

ribosomes released from the membranes were recovered by differential centrifugation in the sediment, ribosomal proteins did not contribute to the value of released proteins in Fig. 9.

e. Effect of Sonication and French Press Treatment on RM

Mechanical disruption is frequently used in attempts to release the content of membrane vesicles

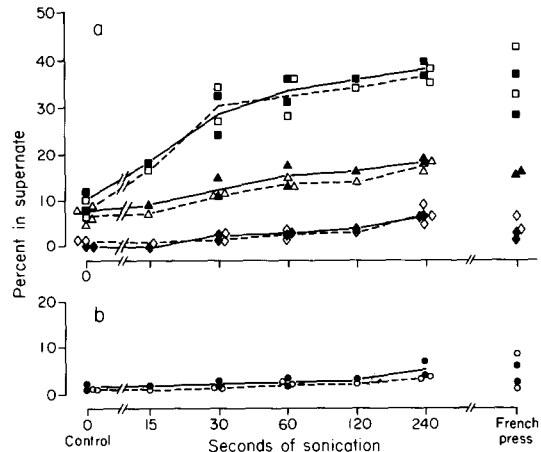


FIGURE 10 Differential effect of sonication and French press treatment on the release of short-term labeled proteins, and NADPH and NADH cytochrome *c* reductases. RM (³H]leucine-30 min, preparation d₃, Table I) were resuspended (3–4 mg protein per ml) in modified LSB or HSB (containing only 10 mM Tris-HCl, pH 7.5). At times indicated in the graph, 1 ml aliquots were removed from a sample sonicated as described in Material and Methods, or from a sample treated with the French press. Sedimentable membranes and ribosomes were separated from supernatants by differential centrifugation (90 min-50K-A321). TCA-precipitable radioactivity (□—□, ■—■), protein (△—△, ▲—▲), and NADH (◇—◇, ◆—◆) and NADPH (○—○, ●—●) cytochrome *c* reductase activities were determined before fractionation, in supernatants and in resuspended pellets. The results are given as percentages as explained in Fig. 9. In nonfractionated untreated RM, the specific activity of NADPH and NADH cytochrome *c* reductase (four experiments) was 97–129 and 200–321 enzyme units per mg protein, respectively. Recoveries of NADPH cytochrome *c* reductase after fractionation of sonicated samples (supernatant plus pellet) were higher than 65%. For NADH cytochrome *c* reductase, a consistently higher recovery was obtained in LSB (>81%) than in HSB (>52%). For both enzymes, recoveries were higher than 80% after French press treatment. Open symbols indicate measurements in LSB and filled symbols in HSB.

(Campbell et al., 1960; Dallner, 1963; Dallman et al., 1969). We therefore examined whether French press and sonication in HSB or LSB had a differential effect on the release of microsomal constituents similar to treatment with low detergent concentrations, Fig. 10 a and b show that this was the case and that short-term labeled (³H]-leucine-30 min) proteins (Fig. 10 a — — □ — —

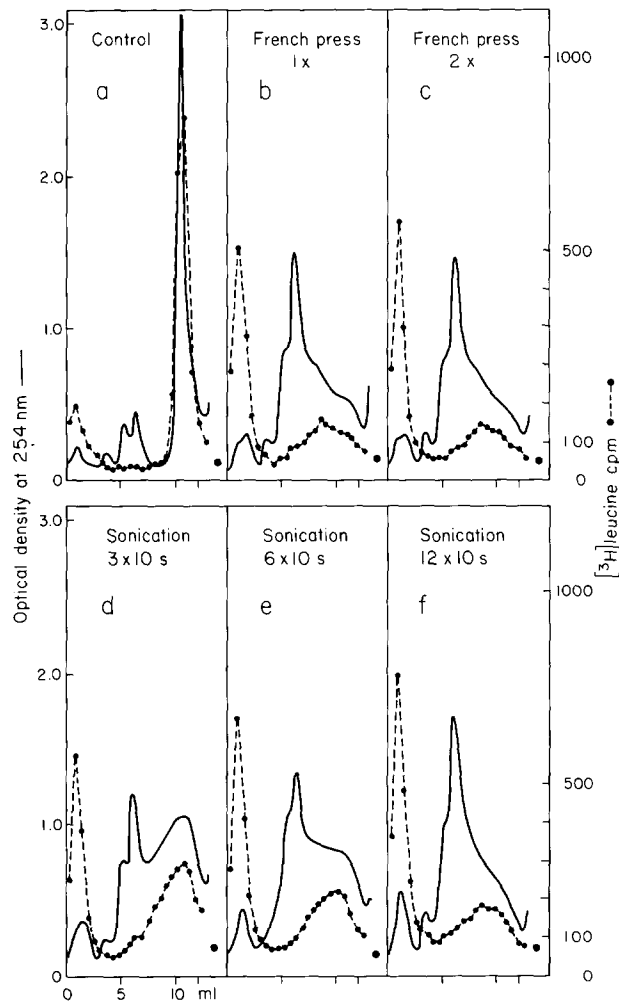


FIGURE 11 Effect of sonication and French press treatment on the release of short-term labeled proteins and on the integrity of RM. RM ($[^3\text{H}]$ leucine-30 min, preparation d_3 , Table I) in HSB (3-4 mg protein per ml) were treated with the French press or sonicated as indicated in each panel of the figure. Aliquots (500 μl) were loaded onto sucrose gradients (10-50% SHSB), which were centrifuged and analyzed as described in the legend for Fig. 1.

and \blacksquare) were extensively released, by treatments which did not affect NADH and NADPH cytochrome *c* reductases (Fig. 10 a and Fig. 10 b, \diamond and \blacklozenge ; \circ and \bullet) which remained bound in media of low (LSB) or high (HSB) ionic strength. Total protein (Fig. 10 a, \triangle and \blacktriangle) followed an intermediary curve of release. Mechanical disruption, however, was not as effective as low detergent concentration treatment in releasing short-term labeled proteins (cf. Figs. 9 and 10). Moreover, mechanical disruption (see density gradient analy-

sis, Fig. 11), effective enough to release $\sim 50\%$ of short-term labeled proteins, brought about pronounced changes in the sedimentation properties of the vesicles. Increased absorbance at the ribosome region also suggested that a partial ribosome detachment was caused, as previously reported by Dallman et al. (1969).

The extent of ribosome release caused by French press treatment was demonstrated on 5-20% sucrose gradients (cf. OD profiles in Fig. 12 c and f), and the effect on the sedimentation properties of the vesicles was assessed in 10-50% sucrose gradi-

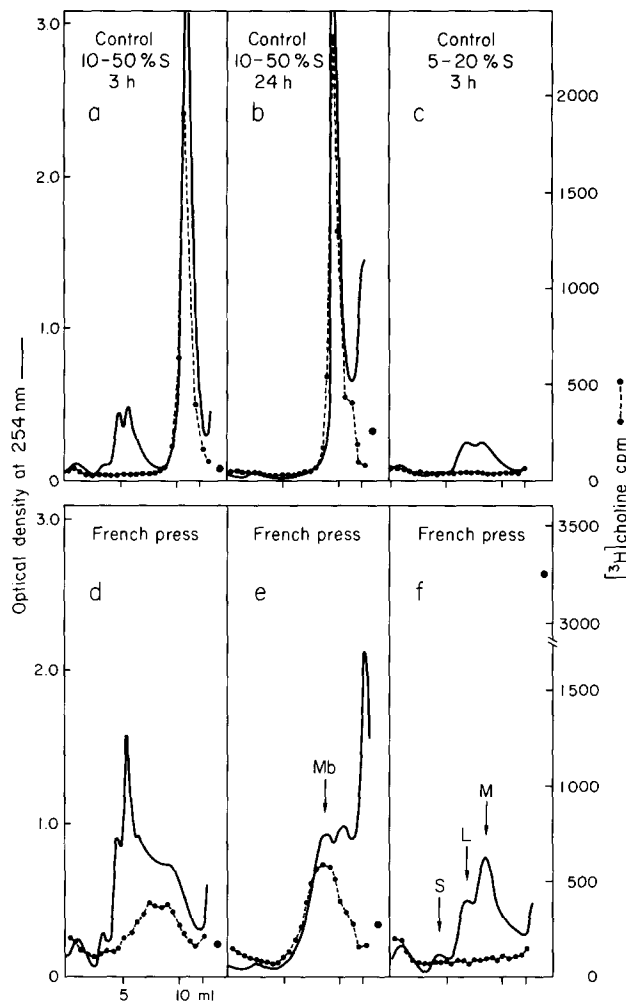


FIGURE 12 Disruption of RM and release of ribosomes caused by French press treatment. RM (3–4 mg protein per ml), containing labeled phospholipids ($[^3\text{H}]$ choline-4 h, preparation a., Table I) were kept at 0°C in HSB as controls (a–c) or passed twice through the French press (d–f). Aliquots (500 μl) were loaded onto 10–50% SHSB gradients (a, b, d, e) or 5–20% SHSB gradients (c, f), which were centrifuged (40K-SB283) for 3 h (a, c, d, f) or for 24 h (b, e). Analysis was carried out as explained in the legend to Fig. 1.

ents (cf. distribution of $[^3\text{H}]$ choline-labeled phospholipids in Fig. 12 a, b, d, e). It was apparent that the membrane band was broader after mechanical disruption (cf. radioactivity distribution in Fig. 12 a and d), and that the average isopycnic density, measured after 24 h of centrifugation (cf. radioactivity distribution in Fig. 12 b and e), was decreased, reflecting mainly size heterogeneity and the extensive ribosome detachment (which affected 45–70% of the bound ribosomes). The radioactivity distribution (Fig. 12 f) shows that

released ribosomes were not bound to membrane fragments. After prolonged centrifugation (24 h), the ribosomes could be sedimented through the membrane region to the bottom part of the gradient (Fig. 12 e).

f. Accessibility of Microsomal Proteins to Added Proteases

Taken together the previous results suggest that short-term labeled proteins ($[^3\text{H}]$ leucine-30 min),

which are preferentially released at low concentrations of detergents or by mechanical disruption, are secretory proteins or proteins segregated within the cavity of the rough microsomes, which represents the cisternal cavity of the ER.

If, in fact, short-term labeled proteins are segregated within the microsomal vesicles, they should be inaccessible to proteolytic enzymes added to the vesicles *in vitro*, for Ito and Sato (1969) have shown that such enzymes are not able to cross microsomal membranes and limit their digestive action to proteins on the outer face of the vesicles. In fact, it was found that the kinetics of digestion of short-term labeled proteins from RM ($[^3\text{H}]$ leucine-30 min, preparation d_2 , Table I) incubated in HSB with a mixture of the proteases trypsin and chymotrypsin was strikingly affected by the presence of 0.025% DOC (Fig. 13). When DOC was not added (—●—, Fig. 13) short-term labeled proteins were extensively protected from digestion, so that only ~15% of the total initial radioactivity became acid soluble after incubation with the proteases for 5 h at 30°C. Leakage of content from some vesicles, which was found to occur even in control microsomes incubated with no enzymes or detergents at 30°C (radioactivity in top

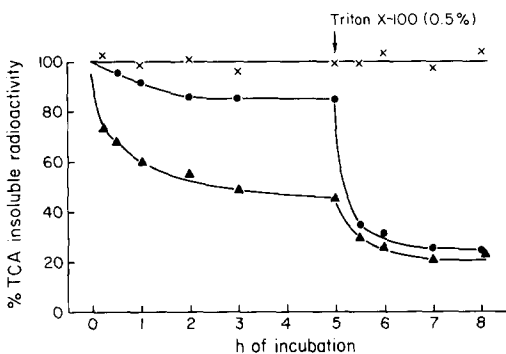


FIGURE 13 Kinetics of digestion of short-term labeled proteins in rough microsomes. RM ($[^3\text{H}]$ leucine-30 min, preparation d_2 , Table I) in HSB (3–4 mg protein per ml), with (▲—▲) or without (x—x, ●—●) 0.025% DOC, received HSB (x—x) or a mixture of trypsin and chymotrypsin in HSB (3 mg of each dissolved just before use in 10 ml) to a final concentration of 50 $\mu\text{g}/\text{ml}$ of each enzyme. During incubation at 30°C, 100- μl aliquots were withdrawn and pipetted onto filter paper discs to measure TCA-precipitable radioactivity. After 5 h of incubation (arrow) all samples received Triton X-100 to a final concentration of 0.5%. Aliquots for zero time values were taken before the addition of enzymes. When necessary, values were corrected for dilution.

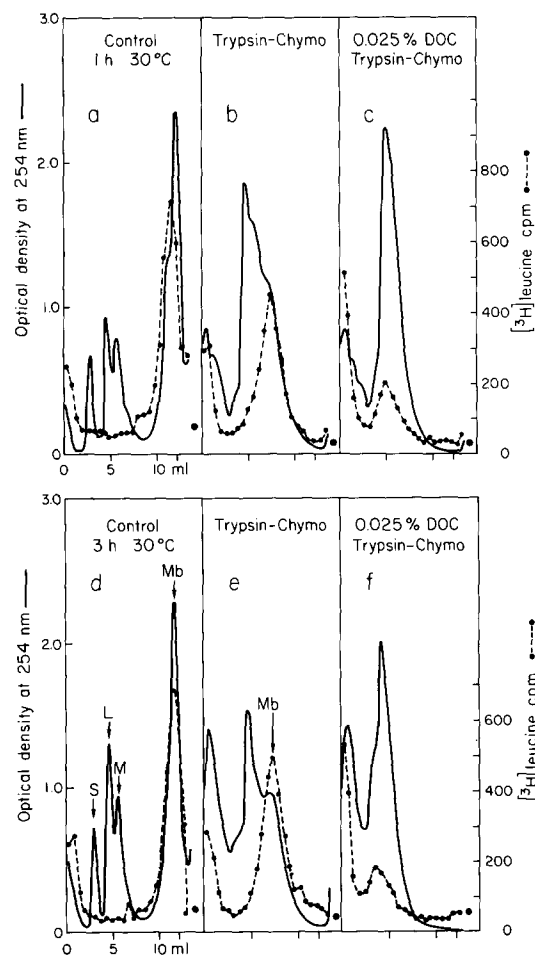


FIGURE 14 Protection of short-term labeled proteins from proteolytic attack. 500- μl aliquots of labeled RM ($[^3\text{H}]$ leucine-30 min) incubated as described in Fig. 13 (same experiment) were withdrawn at 1 and 3 h, loaded onto linear sucrose density gradients (10–50% SHSB), centrifuged, and fractionated as described in the legend to Fig. 1.

part of gradients in Fig. 14 a and d), is sufficient to account for this level of digestion. The acid insoluble radioactivity resistant to proteolysis was still associated with the vesicles, as shown by sucrose density gradient analysis of aliquots taken after 1 and 3 h of proteolysis in the absence of detergent (Fig. 14 b and e). Such radioactivity corresponded to proteins protected by the membranes, which became accessible to the enzymes if 0.5% Triton X-100 was added after 5 h of incubation (Fig. 13, —●—, after arrow). On the other hand, when DOC (0.025%) was present (—▲—,

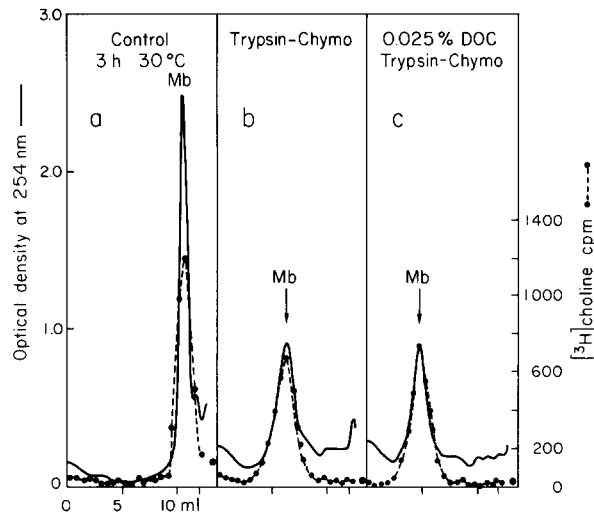


FIGURE 15 Distribution of labeled membrane phospholipid and changes on the density of rough microsomes after proteolysis with trypsin and chymotrypsin. RM ($[^3\text{H}]$ choline-4 h, preparation a, Table I) in HSB (3–4 mg/ml) were incubated as described in the legend to Fig. 13. Aliquots (500 μl) were loaded onto linear sucrose density gradients (10–50% SHSB) which after centrifugation (28 h-40K-SB283) were analyzed as described in the legend to Fig. 1.

Fig. 13) from the beginning of incubation, the digestion proceeded rapidly and reached a level of $\sim 60\%$ after 5 h. A fraction of initial radioactivity remained acid insoluble, even after this time ($\sim 40\%$), but at least half of this was due to labeled peptides, no longer associated with the membrane (Fig. 14 c and f), which were themselves resistant to proteolysis. In fact, $\sim 20\%$ of the initial short-term labeled radioactivity was acid insoluble, even when 0.5% Triton X-100 was added to completely solubilize membranes during proteolysis. Proteolyzed vesicles had a lighter density (radioactive peaks in Fig. 14 b, c, e, and f), as expected from the detachment of ribosomes (Roth, 1960; Lust and Drochmans, 1963; Sabatini and Blobel, 1970) and the degradation of proteins from the outer face of the membranes (Ito and Sato, 1969; Roth, 1960). Similar results were obtained with RM stripped of their ribosomes by the puromycin-KCl procedure, indicating that ribosome removal did not facilitate leakage of content or the entrance of the added proteases into the vesicles.

To further characterize the composition of the residual membranes, proteolyzed vesicles containing labeled phospholipids were also analyzed by isopycnic sedimentation (Fig. 15) after prolonged centrifugation (28 h) to sediment residual ribosomal material. Although the isopycnic density of

the membranes changed during proteolysis, it was clear that protein degradation with or without 0.025% DOC was not accompanied by loss of phospholipid (Fig. 15 and Table III).

As shown in Table III, the total protein content of the vesicles after 3 h digestion without 0.025% DOC was reduced by approximately 45%. 0.025% DOC allowed the digestion of another 21%. This suggests that in addition to proteins on the outside, proteins in the inside face and content of the microsomes were digested.

g. Entrance of High Molecular Weight Polysaccharides into Microsomes

Microsomal membranes are known to be permeable to sucrose (Nilsson et al., 1971). Therefore the presence of large openings in microsomal vesicles should not affect their density in sucrose gradients. On the other hand, high molecular weight polysaccharides do not normally penetrate into microsomes (Wallach and Kamat, 1964; Ito and Sato, 1969). In gradients made of Ficoll or Dextran it should therefore be possible to assess from the isopycnic density of microsomes whether low concentrations of DOC produce membrane openings large enough to allow the passage of these macromolecules. Because of the difficulty in making polysaccharide gradients of the high density

TABLE III
Preservation of Phospholipid in Microsomal Membranes
after Proteolysis⁺

	cpm	
	[³ H]Choline	Protein
	cpm	μg
Control RM, 3 h, 30°C	3095	202
	3266	221
Proteolysis with tryp- sin-chymotrypsin	3063	112
	3103	122
Proteolysis in presence of 0.025% DOC	3021	74
	3120	81

⁺ RM (3 mg protein per ml) prepared from rats which had received an intraperitoneal injection (200 μCi) of [³H]choline (specific activity 480 mCi/mMole) 4 h before sacrifice were incubated for 5 h at 30°C in HSB with a mixture of trypsin-chymotrypsin (50 μg/ml of each). 0.5 ml aliquots were analyzed in sucrose density gradients (10–60%) containing HSB, which were centrifuged for 3 h at 40,000 rpm. Radioactivity and protein were measured throughout the gradients in 100-μl aliquots taken from 0.5 ml fractions.

needed to sediment intact RM isopycally, we used for most of the following experiments RM stripped of ribosomes by the puromycin-KCl procedure (Adelman et al., 1973b). The use of stripped RM also simplified the interpretation of the absorbance profiles because it eliminated or reduced the overlap of membranes with ribosomes. Ribosomes to some extent are always released from RM during centrifugation in HSB and sediment slowly in the high viscosity polysaccharide gradients. Fig. 16 a shows that in sucrose gradients, untreated stripped microsomes sediment isopycally to the same position ($\rho \cong 1.174$) as microsomes treated with 0.025% DOC and that this is the case whether the detergent-treated microsomes are analyzed in gradients containing 0.025% DOC throughout (Fig. 16 c) or only in the load zone (Fig. 16 b).

A striking difference in sedimentation behavior was found when analogous experiments were run in Ficoll (Fig. 17) or Dextran gradients (not shown). Fig. 17 illustrates the case for Ficoll gradients and shows that the isopycnic position depended on whether 0.025% DOC was present or not throughout the gradient (cf. Fig. 17 b and c). If the critical concentration of detergent was only

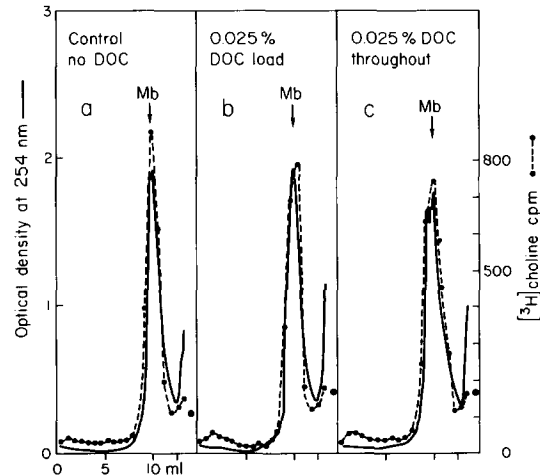


FIGURE 16 Isopycnic sedimentation of stripped rough microsomes in sucrose gradients. Phospholipid labeled ([³H]choline-4 h) RM were used to prepare stripped membranes (Materials and Methods section) which were resuspended (3–4 mg/ml) in HSB, and treated with (b and c) or without (a) 0.025% DOC. Aliquots (500 μl) were loaded onto sucrose density gradients (10–50% SHSB), which in one case contained 0.025% DOC throughout (c). After centrifugation (28 h-40K-SB283) at 3°C, gradients were analyzed as described in the legend to Fig. 1.

present in the loading zone, the isopycnic position of the vesicles, judging from the [³H]choline distribution and absorbance profiles, was the same as in controls ($\rho \cong 1.116$), indicating that permeability changes induced by 0.025% DOC in the sample were reversed once the microsomes entered into the detergent-free gradient. On the other hand (Fig. 17 c), if 0.025% DOC was present throughout the gradient, the vesicles sedimented to the bottom of the tube ($\rho > 1.164$), as indicated by the phospholipid radioactivity in the pellet. In this case only a small amount of residual ribosomes remained within the gradient (Fig. 17 c, OD₂₅₄ absorption profile). The higher isopycnic density of the stripped vesicles in Ficoll gradients containing 0.025% DOC should be expected if the detergent allows penetration of the macromolecular solutes into the vesicle cavity. In this case the buoyant density of the membrane itself should be measured; this density in sucrose gradients was ~ 1.174 . In other experiments, using lower DOC concentrations in the gradient (0.006% or 0.012%), the isopycnic position of the vesicles in polysaccharide gradients was the same as in controls. This was also the case when higher DOC concentrations

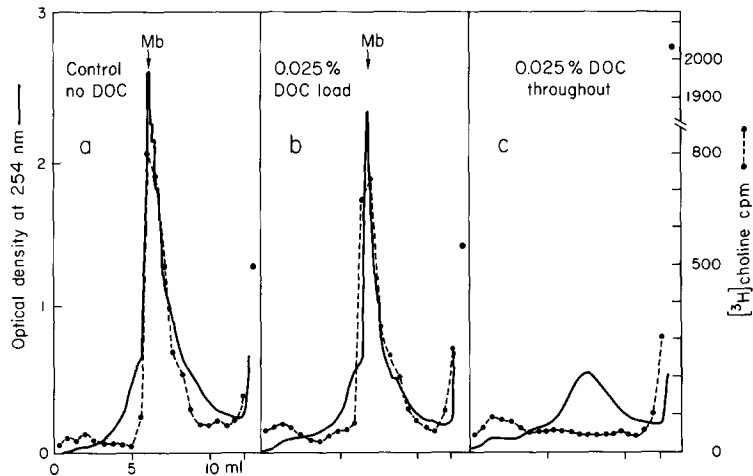


FIGURE 17 Isopycnic sedimentation of stripped microsomes in Ficoll gradients. Aliquots from the same samples used in the experiment described in Fig. 16 were loaded onto linear Ficoll gradients (10–40% Ficoll in modified HSB containing 2 mM $MgCl_2$) which after centrifugation (72 h-40-K-SB283) at 3°C were processed as described in the Materials and Methods section.

were present in the load zone (0.049%), but no DOC was added to the gradient. In fact, in this case the vesicles attained almost the same position as in controls, indicating once more the reversibility of the permeability change induced by detergent.

Since we found (not shown) that when RM treated with 0.025% DOC were analyzed in sucrose gradients, the amount of short-term labeled microsomal proteins ($[^3H]$ leucine-30 min) which remained associated with the vesicles was unaffected by the presence of detergent throughout the gradients, we concluded that release of these proteins from the RM had proceeded to completion before the beginning of gradient centrifugation.

h. Electron Microscopy

Microsomes treated with 0.049% DOC in LSB (Fig. 18 a) or with 0.025% DOC in HSB (Fig. 19 b) and fixed in the presence of DOC did not show visible membrane changes although the mean vesicular diameter was reduced from ~ 3.5 to ~ 2.5 μm . Most vesicles were still bound by a seemingly intact continuous membrane bearing ribosomes on the outer side. Frequently, however, not all ribosomes in the attached polysomes remained in contact with the microsomal membrane. This led to the appearance in detergent-treated microsomes of partially “loose” polysomes

(Fig. 18 a), morphologically similar to those found by other authors (Lee et al., 1971) in cell fractions from mouse sarcoma cells lysed in the presence of 0.13% Triton X-100. On the other hand, membrane openings were easily found in microsomes treated in LSB with higher DOC concentrations ($\geq 0.1\%$) or in microsomes which were treated in HSB with detergent concentrations above 0.05% and fixed in the presence of detergent (Fig. 18 b and c). The proportion of microsomal vesicles with large membrane discontinuities was higher in HSB. Short curved membrane fragments, resembling small open vesicles bearing loose polysomes on one side, were numerous (Fig. 18 b). In the same samples, however, larger and more intact microsomes were also found which only occasionally showed membrane discontinuities (Fig. 18 c). Thus, we failed to detect membrane discontinuities in the electron microscope unless levels of detergent were used which altered the phospholipid and protein composition of the membranes as shown in a previous section. This suggests that the leakage of short-term labeled proteins from microsomes treated with lower detergent concentrations (0.049% in LSB and 0.025% in HSB) occurred through membrane openings which were either unstable or sufficiently small that they were not recognized in sectioned specimens. Lack of membrane discontinuities may also be explained if leakage occurred during a dynamic process involving a detergent-promoted pinching-off and re-

sealing of the original microsomes, which left no morphological vestiges and preceded a more pronounced membrane disassembly. As expected from the biochemical analysis, the earliest morphological changes were found in samples treated with detergents at higher ionic strengths.

Vesicles were also examined after proteolysis in trypsin-chymotrypsin, with or without the addition of a low DOC concentration (0.025%), in an attempt to detect morphological correlates due to the digestion of protein on each side of the membrane (Figs. 19 and 20). Proteolysed vesicles were largely denuded of ribosomes (cf. Fig. 20 and 19 a), but were still closed by a continuous membrane (Fig. 20 a). On the other hand, more pronounced morphological changes and some membrane discontinuities were observed when proteolysis was carried out in the presence of levels of detergent which were insufficient to produce visible openings (cf. Fig. 20 b and Fig. 19 b), but which were found to increase the extent of proteolysis (see section f). Vesicles had variable sizes and shapes but the thickness of the membranes was only slightly reduced by the more extensive protein digestion allowed by the detergent. Under all conditions examined, the typical electron microscopic appearance of the membranes was largely unchanged (cf. inserts to Figs. 19 a, b and 20 a, b).

DISCUSSION

The preceding observations demonstrate that at critical ratios of detergent to microsomal phospholipid, low concentrations of DOC (0.025–0.049%) can induce the reversible formation of membrane pores or openings in liver microsomes without leading to an extensive membrane disassembly. The formation of openings large enough to allow the passage of macromolecules into and out of the vesicle cavities was inferred from changes in the isopycnic density of microsomes (measured in polysaccharide gradients), from the extent of

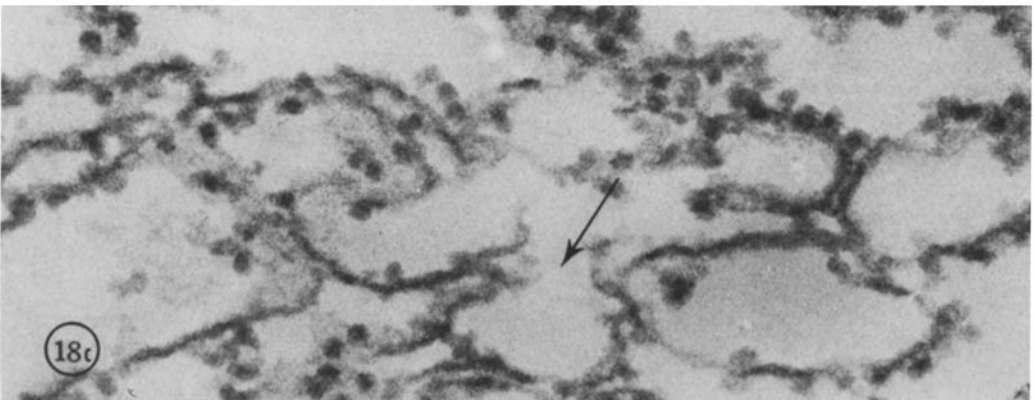
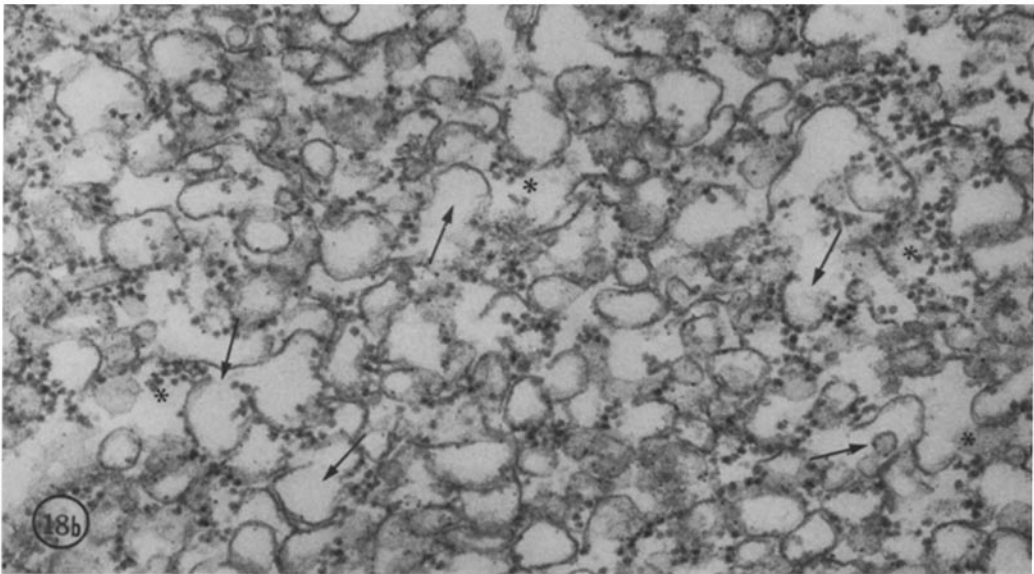
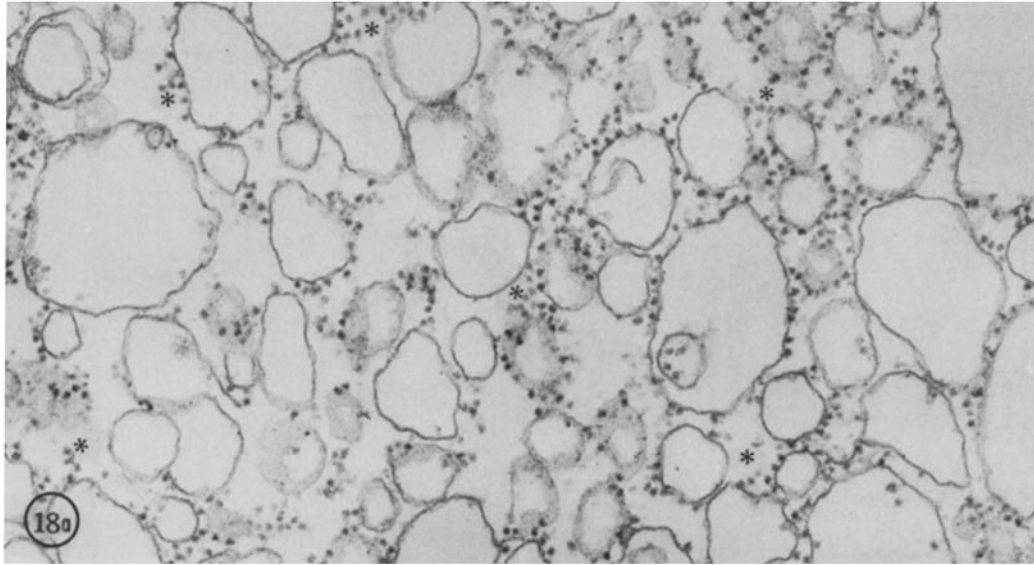
digestion of microsomal proteins produced by exogenous proteases, and from the release of microsomal proteins (thought to represent the luminal content).

Microsomal vesicles treated with low DOC concentrations were shown to have a higher isopycnic density in Ficoll and Dextran gradients containing DOC than in gradients without detergent. In the latter case, the density was the same as that of non-pretreated vesicles. However, in sucrose gradients no changes were detected due to the presence of or pretreatment with DOC. We therefore concluded that the higher isopycnic density in polysaccharide gradients mainly reflected the effect of DOC in allowing large solutes to penetrate into the vesicular cavities, and that this effect was reversible. On the other hand, the composition of the microsomal membranes (judging from the true isopycnic density measured in gradients of sucrose, which is a penetrating solute), appeared not to be greatly affected by the detergent treatment.

Several authors have shown that the barrier which prevents the passage of macromolecules into the vesicular cavities of microsomes remains effective even after proteins in the outer face of the membranes and membrane-bound ribosomes are extensively degraded or removed by proteolysis (Roth, 1960; Ito and Sato, 1969; Sabatini and Blobel, 1970). It is currently thought that a main restriction to the passage of macromolecules across microsomal membranes is imposed by the continuous or quasi-continuous layer of phospholipids, which is a general feature of most biological membranes (see review by Singer and Nicolson, 1972) and is highly sensitive to the action of detergents and lipases. We found that treatment with 0.025% DOC allowed a more extensive action of trypsin and chymotrypsin and permitted the digestion of proteins, such as short-term labeled proteins (labeled after 30 min of radioactive leucine administration), which are largely protected during the

FIGURE 18 a Electron micrograph of rough microsomes resuspended in LSB which received 0.05% DOC and were kept at 0°C for 15 min before fixation. Although most vesicles are devoid of an electron-opaque content, the vesicle profiles are delineated by an apparently continuous membrane. Note that not always do all ribosomes in the attached polysomes remain in direct contact with the ER membranes. Some of the resulting partially "loose" polysomes are indicated by asterisks (*). $\times 51,000$.

FIGURES 18 b and c Rough microsomes which were resuspended in HSB and received 0.1% DOC before fixation. Although membrane discontinuities are numerous (arrows) membrane profiles are still curved, resemble the vesicular shape and have loose polysomes (*) bound only to one membrane side. Fig. 18 b, $\times 51,000$; Fig. 18 c, $\times 128,000$.



digestion of untreated vesicles. This observation strongly suggests that in the presence of 0.025% DOC, the proteases were able to enter into the microsomes and exert their action from both sides

of the ER membrane, as also suggested by Kuriyama (1972). In spite of the extensive digestion, the vesicles retained their intact phospholipid complement and therefore had a low protein to phos-

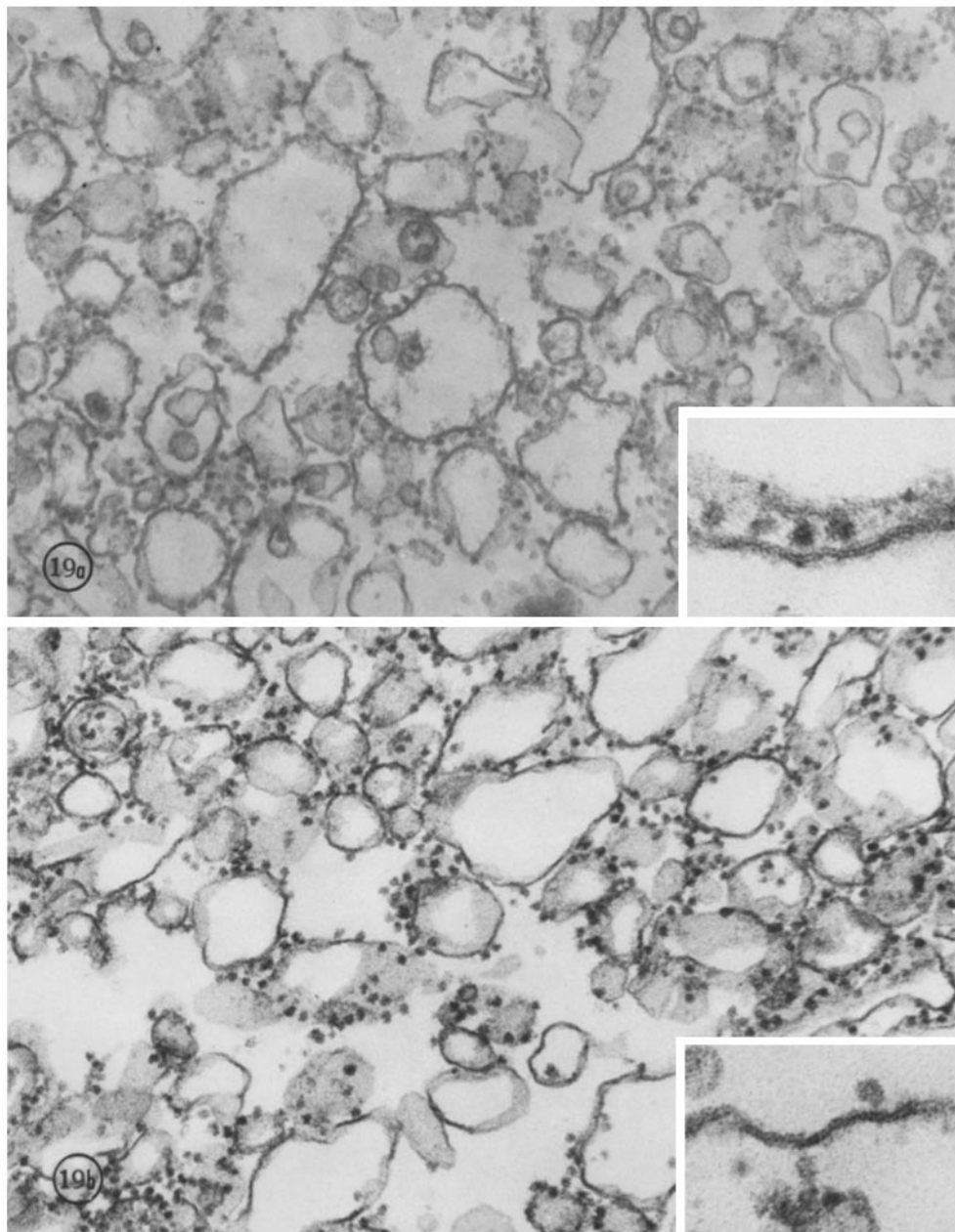


FIGURE 19 a Control rough microsomes incubated at 30°C in HSB for 1 h before fixation in 2% glutaraldehyde. $\times 51,000$.

FIGURE 19 b Rough microsomes incubated at 30°C for 1 h in HSB containing 0.025% DOC before fixation in 2% glutaraldehyde containing DOC. At this low concentration of DOC even in HSB most vesicles appear surrounded by a membrane without visible discontinuities. $\times 51,000$.

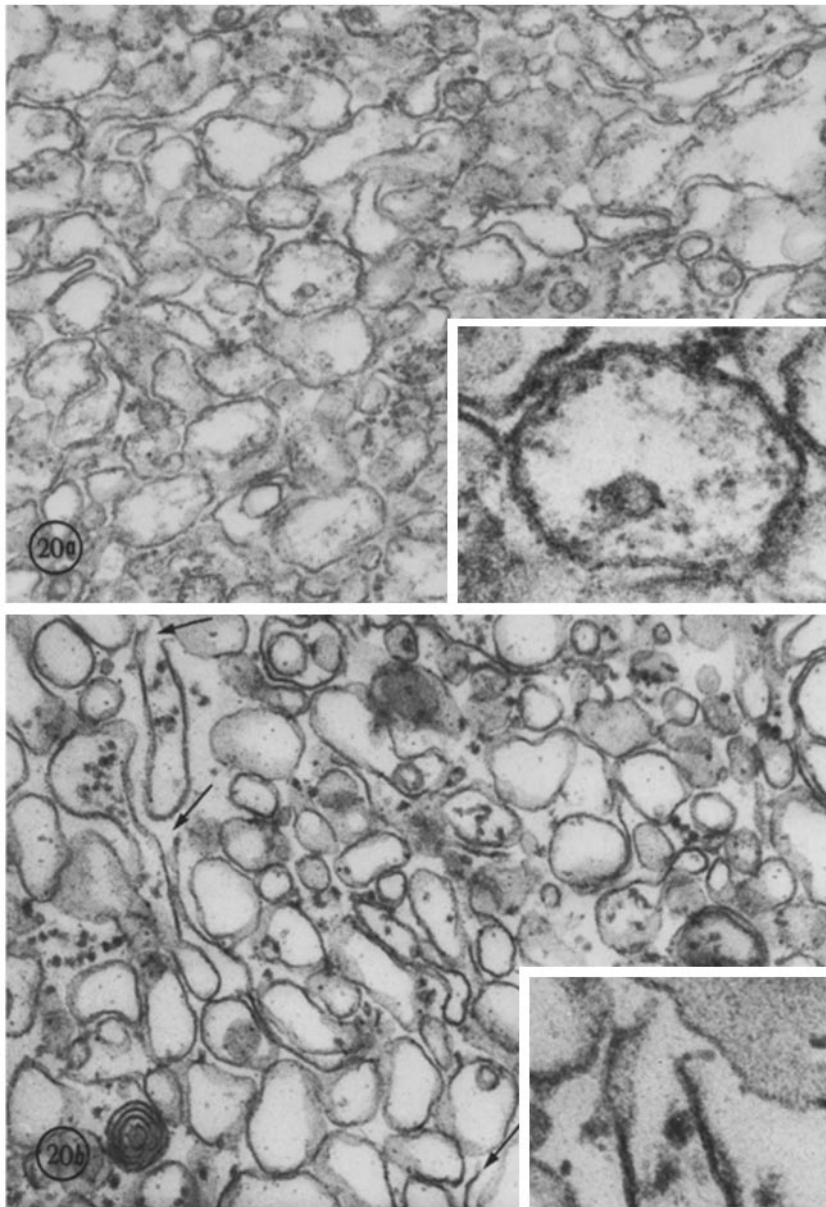


FIGURE 20 a Rough microsomes incubated in HSB at 30°C for 1 h with trypsin and chymotrypsin (50 $\mu\text{g}/\text{ml}$ of each) before fixation in 2% glutaraldehyde. Vesicles are stripped of ribosomes but no membrane discontinuities are observed. $\times 51,000$.

FIGURE 20 b Rough microsomes incubated at 30°C for 1 h in HSB with trypsin and chymotrypsin (50 $\mu\text{g}/\text{ml}$ of each) in the presence of 0.025% DOC, and fixed with a 2% glutaraldehyde solution containing DOC. After digestion changes in the shape and size of vesicles are apparent. Some membrane discontinuities are pointed out by arrows. Numerous detached ribosomes are seen in between the vesicles. $\times 51,000$.

Insets to Figs. 19 and 20 show portions of microsomal membranes chosen from regions with few attached ribosomes. $\times 150,000$.

pholipid ratio and a low isopycnic density. Electron microscopic observations of these vesicles demonstrated that they were bounded by a thinner, but still clearly defined membrane, denuded of ribosomes. The residual protein in these membranes represented approximately 34% of the original protein content. This fraction of the microsomal protein could only be digested by the proteases if levels of detergent higher than 0.098%, which also led to membrane disassembly and to loss of phospholipids, were added to the mixtures. Thus it is possible that the residual membrane protein fraction (resistant to proteolysis in 0.025% DOC) represents proteins buried in the phospholipid layer or fragments from proteins which spanned the whole membrane thickness. If all residual proteins were buried within the membrane, then nonsecretory proteins exposed on the inner microsomal face would contribute less than 10% of the total microsomal protein (Table III). Such protein distribution would correspond to a highly asymmetric membrane structure (see also Steck et al., 1971). On the other hand, RM incubated with trypsin-chymotrypsin up to 5 h at 0°C showed after SDS-disc acrylamide electrophoresis no digestion of proteins from our "content fraction," whereas other proteins from the outside phase of the microsomes are clearly digested (see also Sabatini and Blobel, 1970).

Approximately 10% of the total microsomal protein was released from rough microsomes by the low detergent treatment. Several observations indicated that the released protein fraction represented a specific set of proteins and suggested that they leaked from the vesicular cavities of microsomes. The kinetics of labeling of the bulk of the released proteins was significantly different from that of the total microsomal protein, as expected from proteins originally segregated into the cisternal lumen of the endoplasmic reticulum and destined for secretion. The set of released proteins included 56% of the microsomal proteins of rapid turnover, which could be labeled *in vivo* after 30 min of radioactive leucine administration (short-term labeled proteins), and a major portion (45%) of the microsomal glycoproteins (labeled after similar treatment with [³H]glucosamine (see Fig. 3). As expected from the kinetics of synthesis of secretory proteins (Peters et al., 1971), direct SDS-acrylamide electrophoretic analysis (to be published) demonstrated that serum albumin and other serum proteins were in fact a major fraction within the set of short-

term labeled proteins released by detergent. Recent experiments (in collaboration with Dr. P. Lazarow) showed that more than 80% of pulse-labeled proteins ([³H]leucine-2 min, comparable to preparation e₂, Table I) released by low detergent treatment can be precipitated specifically with antiserum against total rat serum. As mentioned above, proteolysis experiments also indicated that short-term labeled proteins are segregated products, enclosed in a microsomal compartment and largely inaccessible to exogenous proteases unless low concentrations of DOC were added.

Mechanical treatments, such as passage through the French press or sonication, expected to disrupt the vesicles and to provide opportunities for leakage of content, also led to the release of short-term labeled proteins and glycoproteins. These observations agree with previous reports (Campbell et al., 1960; Redman and Cherian, 1972), showing that albumin can be partially released from liver microsomes by sonication. Although in most respects mechanical treatments were found to be qualitatively similar to the low detergent treatment, the former were in general less effective and more difficult to control, leading to protein denaturation and ribosome release. Mechanical disruption has also been shown to be effective in releasing some microsomal enzymes, such as a nucleoside diphosphatase, which is activated during treatment of microsomes with the Turrax blender (Ernster and Jones, 1962; Dallner, 1963). The latter observation is of interest because it has recently been reported (Kuriyama, 1972) that nucleoside diphosphatase is also easily released from microsomes treated with low concentrations of DOC, suggesting that the enzyme is loosely bound to the inside face of microsomes. We have also shown electrophoretically (to be published) that the set of proteins released by DOC is analogous to that released by mechanical disruption. This was found to be true not only for the short-term labeled proteins, but also for a subset of released proteins, which are not extensively labeled during a short-term *in vivo* labeling procedure. Within the latter subset one may expect to find enzymes such as nucleoside diphosphatase, which may be loosely bound to the inside face of microsomes (Ernster and Jones, 1962; Kuriyama, 1972) or proteins which are free and normally reside within the cisternal cavity of the ER.

The concentration of DOC needed to effect the permeability changes indicated by the observations just discussed was insufficient to solubilize

membrane phospholipids or to significantly release several membrane-bound microsomal enzymes, such as NADH and NADPH cytochrome *c* reductases and cytochrome *b₅*, or to detach membrane-bound ribosomes. At two different DOC concentrations (0.196% and 0.049%), a very similar degree of release was attained by changing the microsome concentration so that the ratio of DOC to phospholipid was approximately the same in both cases. The phenomenon of release of content from vesicular structures has been studied extensively with erythrocytes (Seeman, 1967; Bonsal and Hunt, 1971; Seeman et al., 1973; Salton, 1968). For erythrocyte ghosts a preferential release of proteins over phospholipids has been reported (Philippot, 1971) at low DOC concentrations. A selective release of proteins from membranes has also been demonstrated for detergent-treated mitochondrial vesicles (Hall and Crane, 1972), *M. lysodeikticus* (Salton and Nachbar, 1970; Estrugo et al., 1972) and mycoplasma treated with other detergents (Ne'Eman, Kahane and Razin, 1972). Several authors (Kuriyama, 1972; Rostgaard and Moller, 1971; Weihing et al., 1972) have also recently reported the production of membrane pores in detergent-treated microsomes leading to a selective release of proteins.

We cannot exclude the possibility that some true microsomal membrane proteins were solubilized by the low detergent treatment. However, our observations over a wide range of detergent concentrations, with DOC (this paper) and with nonionic detergents, such as Triton X-100 and Brij 35 (to be published), indicate that in all cases the permeability changes and the release of a specific set of proteins preceded membrane lysis as assessed by loss of ribosomes, electron transport enzymes, or by phospholipid solubilization. The stability of the membranes depended on the ionic strength of the medium and was lower in high salt buffer. At low and high ionic strengths, the permeability changes induced by detergent occurred at DOC concentrations lower than the critical micellar level (CMC) which is required to solubilize the phospholipids. In a low ionic strength buffer the CMC is of the order of $1-5 \times 10^{-3}$ M (Small, 1971; Benzonana, 1969). Under these conditions a molar ratio of DOC to phospholipid of 7-8 was required for membrane breakdown, which compares well with values reported for the micellar size of DOC (Benzonana, 1969; Small, 1971). Using labeled DOC, it was possible to estimate that the uptake of more than

one DOC molecule per 40 phospholipid moieties was required to produce the permeability changes. We therefore propose that the uptake of detergent first induced a structural modification within the hydrophobic membrane matrix. This is likely to be a molecular rearrangement or a phase transition within the phospholipid matrix of ER membrane, for example from a lamellar to a micellar distribution, which would account for a less efficient permeability barrier. Lamellar-micellar transitions, such as those described in model systems (Luzzati et al., 1966), have also been proposed to explain the effects of detergent on the permeability of plasma membranes from intact cells (Malenkov et al., 1967; Hodes et al., 1969) and to account for the effect of lysolecithin which causes leakage of Ca^{++} from sarcoplasmic reticulum vesicles (Agostini and Hasselbach, 1971) and, in several systems, promotes membrane fusion before lysis (Lucy, 1970; Poole et al., 1970). It is interesting to note that membrane openings were detected by electron microscopy only in vesicles treated with levels of detergent near the CMC, and therefore sufficient to partially release membrane phospholipids. At lower levels, which produced permeability changes only, a smaller average vesicle diameter was detected, suggesting that vesicle fragmentation and fusion of fragmented membranes occurred reversibly in the presence of detergent. Although previous reports had indicated that it is possible to separate ribosomes from membranes by low detergent concentrations (Ernster et al., 1962), we have been unable to extensively release ribosomes from rough microsomes without a concomitant partial phospholipid solubilization. Although some ribosomes were detached leading to the formation of partially "loose" polysomes, our results suggest that the receptor for active ribosomes is also a structural component, at least partly embedded in the membrane.

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