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Purification of Hepatic Microsomal Membranes†

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ABSTRACT: A method is described for the isolation in high yields of apparently intact microsomal membranes. Solutions of high ionic strength are used to remove attached ribosomes and large amounts of protein, including serum protein, which may be adsorbed to the outer surface of the membranes. Subsequent extraction with dilute sodium deoxycholate tends to release preferentially small quantities of protein probably retained within the closed vesicles. Several lines of evidence suggest that the membranes are relatively free of extraneous protein after these purification steps. The isolated membranes, in contrast to the originally heterogeneous vesicles from which they are derived, exhibit a highly uniform buoyant density. This observation supports earlier suggestions that the membranes of smooth and rough endoplasmic reticulum are structurally similar.

As attention focuses on the detailed molecular properties of biological membranes, it becomes increasingly important not only that membranes can be isolated in a highly purified state but also that their purification can be achieved without altering the properties responsible for their normal structural and functional role in the cell. In this regard none of the methods commonly used for the purification of hepatic microsomal membranes seem to be completely satisfactory.

Microsomal membranes that are free of attached ribosomes and other morphologically detectable cell components have been prepared by methods utilizing differential and density gradient centrifugation to isolate the preexisting smooth microsomal vesicles (Chauveau *et al.*, 1962; Dallner, 1963; Glauman and Dallner, 1968). Because of the mild conditions of isolation, these methods insure recoveries of relatively intact membranes but do not remove serum proteins apparently contained within the closed vesicles (Peters, 1962) nor, with the conditions of low ionic strength normally employed, would they be expected to remove adsorbed cytoplasmic proteins.

The membranes of the rough as well as smooth microsomal vesicles have been purified by methods utilizing chemical, enzymatic, and mechanical treatment to remove the membrane-attached ribosomes and the contents of the vesicles. As discussed in a review by Dallner and Ernster (1968), several of these methods (*e.g.*, extraction with EDTA, exposure to hypotonic solutions, sonication) do not appear to be very effective. More recently treatment of microsomes with trypsin (Omura *et al.*, 1967), 2 M lithium chloride (Scott-Burden and Hawtrey, 1969), or 0.25 M Tris (pH 8) followed by sonication (Glaumann, 1970) have been shown to remove membrane attached ribosomes. These treatments, however, do not seem to completely release the contents from the vesicles, and Tris sonication also does not completely release RNA. On the other hand, treatment with 0.26% sodium deoxycholate seems to effectively release both ribosomes and contents (Ernster *et al.*, 1962), and for this reason has been widely adopted as a method for purifying the membranes (Omura *et al.*, 1967) and also for separating and identifying the contents of the vesicles (Redman, 1967). This treatment, however, also releases considerable quantities of phospholipid from the sedimenting vesicles and it, as well as trypsin digestion, also releases NADPH–cytochrome *c* reductase and cytochrome b_5 (Ernster *et al.*, 1962), two presumably membrane linked proteins (Omura *et al.*, 1967). These observations, as well as

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others to be presented in this report, suggest that the membranes may be considerably altered by this isolation procedure.

In this report it is shown that conditions of high ionic strength and pH will release the membrane-attached ribosomes and large amounts of apparently adsorbed cytoplasmic proteins from the hepatic microsomal vesicles without any apparent alteration of the membrane itself. These conditions are similar to those used in an early study by Simkin and Work (1957) to resolve microsomes into RNA and phospholipid-rich subfractions whose morphological significance was not until now clearly understood. Additional protein, apparently contained within the closed vesicles, is preferentially released during subsequent extraction with dilute sodium deoxycholate. It is also shown that the membranes isolated by this method exhibit very little of the physical or morphological heterogeneity displayed by untreated hepatic microsomal vesicles (Rothschild, 1963; Dallner *et al.*, 1968).

Methods

Isolation of Liver Microsomes. Livers from male Carsworth CFE rats were homogenized in 10 ml of 0.25 M sucrose/g (wet weight) of liver. Other details of tissue preparation and homogenization have been described previously (Manganiello and Phillips, 1965). All operations were carried out at 0–4°. Cell debris, nuclei, and mitochondria were removed by centrifugation at 14,000 rpm for 15 min in an International 865 rotor. Microsomes were sedimented at 40,000 rpm for 20 min in a Spinco 40 rotor.

Salt Extraction of Liver Microsomes. Each crude microsomal pellet from 1.2 g of liver was suspended by homogenization in 5 ml of 0.14 M sodium chloride. Suspensions of two pellets were combined, diluted to 11 ml with 0.14 M sodium chloride, and centrifuged at 40,000 rpm for 20 min in a Spinco 40 rotor. The resulting pellet was suspended by homogenization in 11 ml of 0.14 M sodium chloride and recentrifuged. This operation was repeated twice with 1.0 M sodium chloride and finally once with 0.1 M $NaHCO_3$–0.1 M Na_2CO_3. In order to obtain a firm pellet, the duration of centrifugation for the bicarbonate extraction was increased to 30 min. In more recent experiments, single microsomal pellets from 1.2 g of liver were extracted only once with each of the salt solutions at a final volume of 11 ml. This modification does not appear to alter significantly the chemical and enzymatic properties of the final preparation.

Assay of Cytochromes and Enzymes. The presence in microsomes of bound hemoglobin seriously interferes with the measurement of cytochrome b_5 and the microsomal CO-binding cytochrome. Therefore, in experiments in which these two hemochromogens were measured, the bound hemoglobin was reduced by perfusion of the intact liver through the portal vein with 50 ml of ice-cold 0.9% sodium chloride.

The content of cytochrome b_5 was determined from the difference spectrum (dithionite reduced minus oxidized) of microsomal fractions suspended in 0.1 M Tris (pH 7.6). It was assumed that the $E_{410-425}$ is 170 cm^{-1} mM^{-1} (Strittmatter and Velick, 1956). The content of the CO-binding cytochrome was measured from the CO difference spectrum of dithionite-reduced suspensions of liver microsomes in 0.1 M Tris (pH 7.6)–0.3% sodium deoxycholate using $E_{420-490}$ of 111 cm^{-1} mM^{-1} (Omura and Sato, 1964). Difference spectra were recorded on a Bausch and Lomb Spectronic 505 recording spectrophotometer. The glucose-6-phosphatase activity of microsomal fractions was measured in a reaction mixture containing 20 μmoles of potassium maleate buffer (pH 6.5) and 25 μmoles of potassium glucose 6-phosphate adjusted to pH 6.5 in a final volume of 0.5 ml. Other conditions of assay were as described by Swanson (1955). NADH and NADPH–cytochrome *c* reductase were assayed as described earlier (Manganiello and Phillips, 1965).

Immunochemical Assay of Serum Protein. The serum protein content of microsomal subfractions was determined by immunoprecipitation with rabbit antiserum to rat serum essentially as described by Peters (1962). Microsomal subfractions containing 100–150 μg of protein were dissolved in 0.5 ml of 0.9% sodium chloride–0.12% sodium deoxycholate (pH 7.8) and after the addition of 8.5 μg of carrier rat serum protein were treated with 0.1 ml of the antiserum (purchased from Hyland Laboratories and centrifuged immediately prior to use). Other conditions of precipitation and the washing of the immunoprecipitates were carried out exactly as described. The serum protein content of the subfractions was calculated by comparing the amount of washed immunoprecipitate to a standard curve obtained by immunoprecipitation under identical conditions of increasing amounts of rat serum protein. The addition of carrier rat serum protein was required, particularly with microsomal subfractions of low serum protein content, to give amounts of immunoprecipitate that fall in a linear region of the standard curve.

Chemical Methods. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Phospholipid and RNA were determined as described previously.

Electron Microscopy. Microsomal pellets, suspended in a small volume of the medium to which they had last been exposed, were fixed by the addition of an equal volume of 12.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The suspensions were mixed and allowed to stand for 20 min. Aliquots were placed in Beckman microfuge tubes and centrifuged for 20 min in the microfuge operated at 120 V. The supernatant fluid was decanted and replaced with fresh 6.25% glutaraldehyde solution. The volume of the sample was adjusted to give microsomal pellets about 0.5 mm thick. All samples were prefixed for a total of 2 hr in glutaraldehyde. The pellets were washed, fixed for 1–1.5 hr in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2), dehydrated by passage through an ethanol series, and embedded in Epon 812. Fixation and dehydration procedures were carried out at 0–4°. The pellets were sectioned with a diamond knife; the sections were stained with 1% uranyl acetate for 30 min at 60° and then with lead citrate. They were examined in a Siemans Elmiskop I.

Results

Purification of Microsomal Membranes by Multiple Salt Extraction. The relative amounts of RNA, protein and phospholipid released from the microsomal vesicles by multiple salt extraction are shown in Table I. The RNA is released in two distinct stages. Approximately half is released during the first extraction with 1.0 M sodium chloride, and the remaining RNA, which resists further extraction by this solution, is almost completely released by alkaline sodium carbonate. Stepwise release of protein at each stage of the extraction procedure is also observed. Most important, however, is that the complete extraction sequence removes over 90% of the microsomal RNA and nearly half the protein from particles that retain at least 85% of the total microsomal phospholipid.

The activities of several enzymes and cytochromes in the microsomes before and after salt extraction are shown in Table II. A comparison of total recovered activities indicates

TABLE I: Extraction of Microsomal Protein, Phospholipid, and RNA by a Series of Neutral and Alkaline Salt Solutions.

Fraction		% of Total Recovered		
		Protein	Phospholipid	RNA
First	0.14 M NaCl extract	22.3 (19.2–25.8)[a]	2.2 (1.0–4.7)[a]	5.8 (4.0–8.7)[a]
Second	0.14 M NaCl extract	3.6 (3.0–4.2)	2.0 (1.5–3.9)	2.4 (1.2–4.1)
First	1.0 M NaCl extract	13.8 (12.0–18.7)	4.1 (3.8–7.8)	41.8 (35.2–46.2)
Second	1.0 M NaCl extract	2.7 (2.3–3.3)	0.7 (0–2.2)	1.3 (0.5–2.2)
	0.1 M $NaHCO_3$–0.1 M Na_2CO_3 extract	5.9 (5.0–7.5)	3.0 (0–10.2)	43.2 (36.4–54.8)
	(Salt-extracted vesicles)	52.0 (48.4–54.6)	87.9 (82.5–96.4)[b]	7.0 (6.8–7.3)
		mg/g of liver		
Amount recovered in all fractions		27.2 (20.9–33.0)	12.3 (8.3–18.0)	1.74 (0.9–2.3)

[a] Values represent average and range of 4 separate experiments. [b] In a series of 10 separate experiments the salt-extracted vesicles contained as an average 0.82 mg of phospholipid/mg of protein. The range was 0.67–1.05 mg of phospholipid/mg of protein.

TABLE II: Levels of Enzymes and Cytochromes in Microsomes before and after Repeated Salt Extraction.

	Sp Act. (Units/mg of Protein)		Ratio Salt Extracted: Untreated	Total Act. (Units/g of Liver)		Ratio of Sp Act. Cor[d] Salt Extracted: Untreated
	Untreated	Salt-Extracted		Untreated	Salt Extracted	
Glucose-6-phosphatase[a]	1.5	2.6	1.7	38	45	1.5
	1.7	2.7	1.6	42	44	1.5
	1.4	2.3	1.6	23	25	1.5
NADH–cytochrome *c* reductase[b]	0.46	1.02	2.2	8.1	11.5	1.6
	0.28	0.75	2.7	4.6	8.0	1.7
NADPH–cytochrome *c* reductase[b]	0.17	0.31	1.8	4.4	5.5	1.5
	0.25	0.31	1.2	5.5	4.4	1.6
Cytochrome b_5[c]	0.39	0.76	2.0	9.6	9.1	
	0.46	0.67	1.5	11.9	10.1	
Cytochrome P420[c]	0.61	0.99	1.6	15.2	12.5	
	0.65	0.95	1.5	17.9	14.8	

[a] Units expressed as μmoles of P_i released per 10 min. [b] Units expressed as μmoles of cytochrome *c* reduced per min. [c] Units expressed as nmoles of cytochrome. [d] Corrected for assumed activation or inactivation of enzymatic activity by dividing ratio of specific activity by ratio of total activity of salt extracted to untreated microsomes.

that each of these proteins strongly resists extraction by the salt solutions. In the case of NADH–cytochrome *c* reductase and to a lesser extent NADPH–cytochrome *c* reductase and glucose-6-phosphatase, recoveries of total activities after salt extraction often exceed 100%. If, when comparing specific activities, this activation is taken into account (see last column of Table II), it can be estimated that the amount of each enzyme, and also cytochrome, relative to protein has increased between 1.5- and 2.0-fold during the extraction steps. This range agrees rather well with the average increase of 1.7-fold in the amount of phospholipid relative to protein indicated by the experiments in Table I.

Electron micrographs of the microsomes at different stages of salt extraction are shown in Figure 1. As observed many times previously the untreated microsomes appear as closed, single membrane-limited vesicles, most of which bear attached ribosomes. Figure 1b–d shows that the membranes continue to remain as closed vesicles at each stage of salt extraction. Many of the vesicles are observed to be collapsed after extraction with the isotonic and 1 M solutions of sodium chloride but then appear to return to their original distended shape after the final extractions with the dilute bicarbonate solution. These changes, which probably reflect net movement of water from and into the vesicles in response to changing osmotic gradients (Wallach *et al.*, 1966), provide additional evidence that the membranes remain as intact closed vesicles throughout the extraction procedure.

Morphologically intact ribosomal particles are no longer observed in microsomes that have been extracted with 1 M sodium chloride (Figure 1c). In addition, analytical ultracentrifugation of microsomes in 1 M sodium chloride (results not shown) also indicated an essentially complete absence of sedimenting particles corresponding to the monomeric or dimeric forms of unattached ribosomes. It is apparent

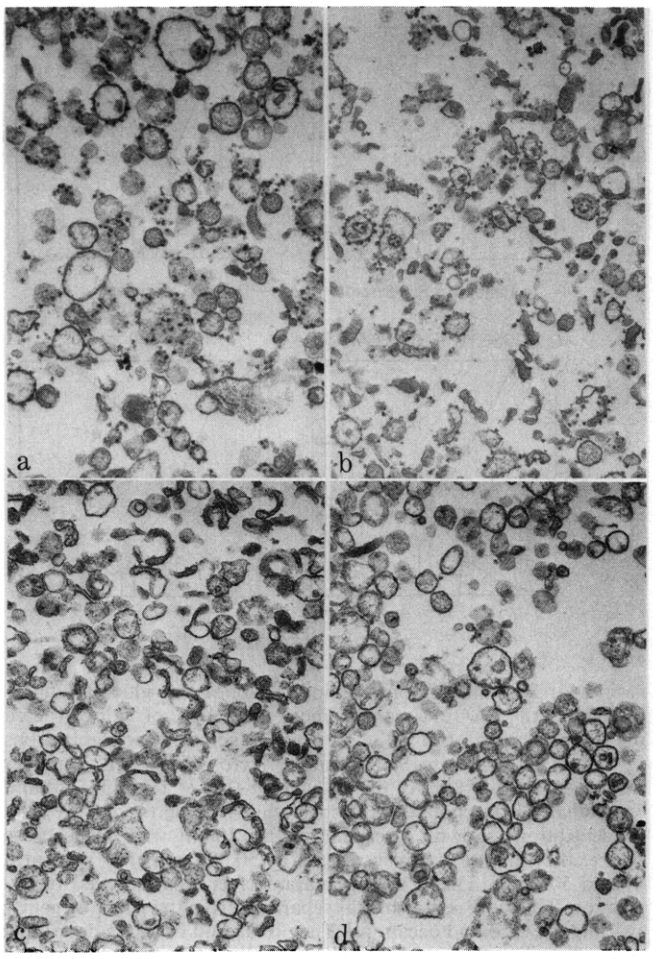

FIGURE 1: Electron micrographs of microsomal pellets at various stages during the salt extraction procedure. (a) Untreated microsomes; (b) microsomes extracted twice with 0.14 M sodium chloride; (c) then twice with 1.0 M sodium chloride; (d) finally twice with 0.1 M sodium carbonate–bicarbonate. Magnification 65,000×.

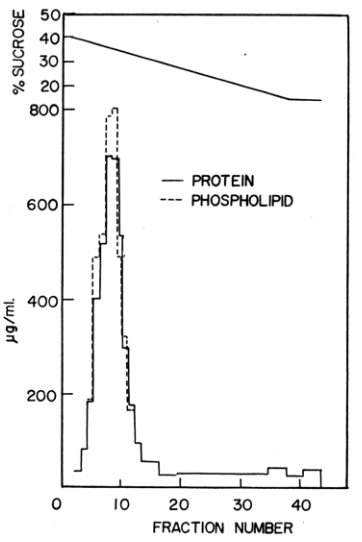

FIGURE 2: Distribution of protein and phospholipid of salt-extracted microsomes after sucrose density gradient centrifugation. The salt-extracted microsomes from 2 g of liver were suspended by homogenization in 5 ml of 0.05 M Tris (pH 7.6) and a 0.5-ml aliquot was layered on a 28-ml 10–40% linear sucrose gradient and centrifuged at 25,000 rpm for 4 hr in a Spinco SW 25.1 rotor. Fractions were assayed for protein and phospholipid as described in the Experimental Section. The position of the peak at 35% sucrose (density = 1.15 at 4°, the approximate temperature of centrifugation) remained unchanged in duplicate tubes that were centrifuged for 8 hr.

from these observations that breakdown of the ribosomal particles, rather than the simple detachment of the intact particles from the membranes, takes place during the extraction procedure. Evidence to be presented in a subsequent communication indicates that nascent ribosomal protein is also released from the microsomal pellet at this step.

It has been consistently observed that the outer surface of the vesicles is slightly more granular in appearance after extraction with 1 M sodium chloride than after the preceding or subsequent extraction steps. Since the appearance of these granules coincides with the breakdown of the ribosomes, and their disappearance with the final release of RNA from the pellet, it is possible that the granules and the RNA resistant to extraction with sodium chloride represent membrane-bound fragments of the ribosomal particles. Although Sabatini *et al.* (1966) have presented evidence that EDTA selectively releases the 30S ribosomal subunit, leaving the large 50S subunit attached to the membranes, we have been unable to demonstrate the presence of either subunit in sodium deoxycholate extracts of the sodium chloride treated microsomes. It is possible that the sodium chloride, in contrast to the EDTA, renders these subunits sensitive to further degradation.

Buoyant Density of Salt-Extracted Vesicles. The salt-extracted vesicles exhibit a highly uniform buoyant density as indicated by the sharp coinciding peaks of protein and phospholipid that are consistently observed when the vesicles are centrifuged to equilibrium in sucrose density gradients (Figure 2). The heterogeneity of the microsomal vesicles after initial extraction with 0.14 M sodium chloride and the change to a lower more uniform density after the subsequent extraction steps are shown in Figure 3. Comparison of density distributions during these extraction steps indicates that the vesicles of high density shift completely to a lower buoyant density whereas the vesicles originally of lowest density seem to remain relatively unchanged. These changes suggests that much of the protein, as well as RNA, released by salt extraction were associated with the denser vesicles, which appear to be predominately of the rough surfaced form (Rothschild, 1963).

Although the change toward a more uniform buoyant density could be due to a salt-induced aggregation of the microsomal vesicles (Dallner and Nilsson, 1966), several lines of evidence suggest that this effect is not of major importance. If random aggregation of heavy and light fractions of rough and smooth vesicles had occurred during the initial removal of protein (after 0.14 M NaCl), the vesicles would be expected to merge into a narrow peak of average buoyant density. As stated above, this behavior was not observed. Furthermore, in experiments not shown, it was observed that peaks of essentially identical low buoyant densities were formed when microsomal subfractions consisting predominately of rough and smooth surfaced vesicles, prepared as described by Manganiello and Phillips (1965), were subjected to the salt extraction procedure. Secondly the lack of gross aggregation is indicated by the observation that longer periods of centrifugation are required for complete sedimentation of salt-washed vesicles than for untreated microsomes (see Methods section). Unfortunately, highly heterogeneous sedimentation velocities of the untreated and salt-washed microsomes obscured efforts

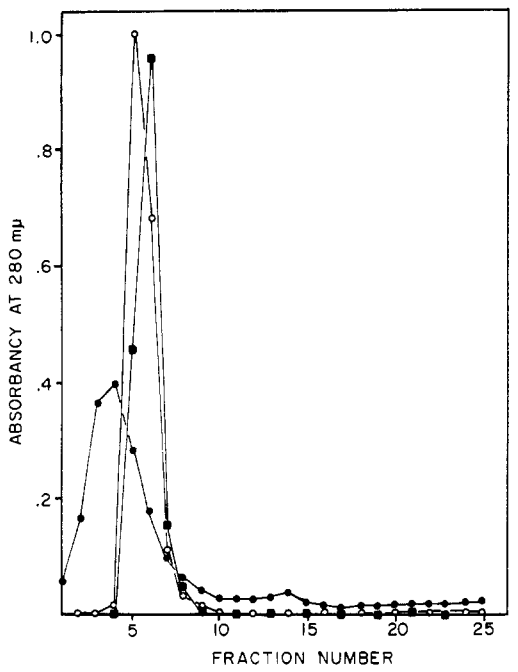

FIGURE 3: Buoyant density of microsomes at various stages in the salt-extraction procedure. Microsomal pellets were prepared and centrifuged as described in Figure 2 except that duration of centrifugation was 14 hr. Fractions were diluted 10-fold before measuring the absorbancy at 280 mμ. The three peaks from left to right, in order of decreasing density, represent the distribution of microsomes washed twice with 0.14 M NaCl (●), then twice with 1.0 M sodium chloride (○), and finally twice with carbonate–bicarbonate (■).

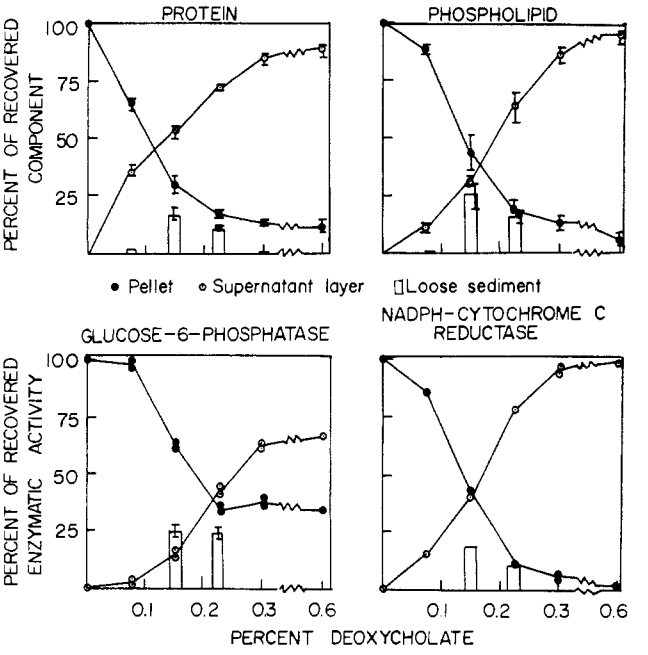

FIGURE 4: Distribution of protein, phospholipid, glucose-6-phosphatase, and NADPH–cytochrome *c* reductase of salt-extracted microsomes centrifuged in the presence of increasing concentrations of sodium deoxycholate. Salt-extracted microsomes from 2 g of liver were suspended in 8 ml of 0.25 M sucrose–0.05 M Tris (pH 7.6) and 3% sodium deoxycholate was added while stirring to give the final concentrations shown. After dilution to 10 ml with the sucrose buffer, the mixture was centrifuged at 50,000 rpm for 1 hr in a Spinco 50 rotor. The clear supernatant layer was removed by aspiration. The loose sediment was separated from the pellet by gently swirling the tube and decanting. The points and brackets describing the distribution of protein and phospholipid are the average and range of three separate experiments.

to make a more direct comparison of their sedimentation behavior. Finally, electron micrographs of salt-extracted vesicles did not reveal the presence of tightly packed clusters of microsomal vesicles that would be expected if strong aggregation had occurred. It therefore seems reasonable to postulate from these experiments that salt extraction of the heterogeneous microsomal vesicles has exposed a membrane core of uniformly high phospholipid content.

Solubilization of the Salt-Extracted Vesicles with Sodium Deoxycholate. The solubilization of the protein, the phospholipid and the glucose-6-phosphatase and NADPH–cytochrome *c* reductase activities of salt-extracted vesicles by increasing concentrations of sodium deoxycholate is shown in Figure 4. The lowest concentration of sodium deoxycholate (0.075%) releases about 35% of the protein from the sedimenting vesicles but only 15% or less of the phospholipid and the microsomal enzymes, whereas at intermediate concentrations of the detergent an essentially parallel release of each of these components is observed. This observation suggests that approximately one-quarter of the protein of the salt-extracted vesicles may be in a form that is less firmly associated with the vesicles than the phospholipid or the enzymes.

At high detergent concentrations (0.3–0.6%) 10% of the protein and a large, apparently distinct fraction of the glucose-6-phosphatase do not become soluble. In a separate series of experiments (Table III) it was observed that 35–45% of the total glucose-6-phosphatase was recovered in the detergent insoluble residue and that its specific activity exceeded by at least 10-fold that of the untreated microsomal pellet. By these criteria the enzyme seems to be in a much more highly purified form than has been obtained previously (Nordlie and Arion, 1964; Segal and Washko, 1957). The detergent-insoluble fraction does not exhibit enhanced specific activities of either NADPH- or NADH–cytochrome *c* reductase, nor is it highly enriched in phospholipid.

At the intermediate concentrations of sodium deoxycholate there is formed, in addition to the firm pellet and clear supernatant layer, a loose red sediment corresponding to the "M" or "membrane fraction" isolated by Ernster *et al.* (1962), and subsequently Dallner (1963). The data in Figure 4 indicate that this fraction is enriched relative to protein in phospholipid and glucose-6-phosphatase, but not in NADPH–cytochrome *c* reductase. In this respect, the fraction resembles the "M" fraction of these workers. On the other hand, not previously noted is the fact that the loose sediment is formed only over a narrow intermediate range of detergent concentrations. It is not observed when washed microsomes are sedimented in the presence of 0.075% deoxycholate and progressively smaller quantities of protein and phospholipid are recovered in this fraction as the concentration of the detergent is increased above 0.15%. A similar pattern was observed for the formation of the loose sediment when unwashed microsomes were centrifuged in the presence of increasing concentrations of deoxycholate, except that it was displaced toward higher detergent concentrations. Again formation of the loose sediment was observed to occur to a significant extent only at intermediate stages in the dispersion of membranes by the detergent. These data, as well as evidence discussed in the introduction, suggest that the "M" fraction may represent microsomal membranes that have been partially disrupted by the detergent.

TABLE III: Distribution of Protein, Phospholipid, Glucose-6-phosphatase, and NADH–Cytochrome *c* Reductase in Deoxycholate-Soluble and -Insoluble Fractions of Salt-Washed Microsomal Membranes.

	Protein (mg/g of Liver)	Phospholipid (mg/g of Liver)
Salt-washed microsomes	17.0 (14.0–19.6)[a]	
0.3% Deoxycholate supernatant layer	16.5 (13.7–18.8)	10.4 (9.3–11.4)[a]
0.3% Deoxycholate pellet	2.7 (1.4–3.8)	1.6 (1.2–2.1)

	Glucose-6-phosphatase		NADH–Cytochrome *c* Reductase	
	Units/mg of Protein	Units/g of Liver	Units/mg of Protein	Units/g of Liver
Salt-washed microsomes	2.6 (2.3–2.8)[a]	42 (38–45)[a]	0.8 (0.6–1.0)[a]	9.8 (8.0–11.5)[a]
0.3% Deoxycholate supernatant layer	2.0 (1.6–2.9)	31 (26–40)	1.3 (1.1–1.5)	16.6 (12.5–22.0)
0.3% Deoxycholate pellet	9.9 (5.9–18.0)	23 (15–28)	0.4 (0.3–0.6)	1.2 (0.4–2.4)

[a] Values refer to average and range of three separate experiments. For definition of units, see Table II.

Although the salt-washed microsomal pellet consists predominantly of closed vesicles (Figure 1d), after extraction with 0.075% deoxycholate the vesicles are predominantly in an open form (Figure 5a). This observation suggests that the protein released by 0.075% deoxycholate may represent protein that was trapped within the closed vesicles. The glucose-6-phosphatase-rich pellets from microsomes treated with 0.3% deoxycholate also appear as open vesicles, which on the average are slightly larger than those seen in the original microsomal pellet (Figure 5b).

Distribution of Microsomal Serum Proteins during Salt and Deoxycholate Extractions. The immunochemically detectable serum protein of hepatic microsomes is resistent to extraction with 0.14 and 1.0 M sodium chloride (Table IV). Although most of this protein is removed by extraction with bicarbonate, complete removal is not obtained until after the subsequent extraction with 0.075% deoxycholate. Since this protein is thought to be contained within the closed vesicles, these observations suggest that bicarbonate may be responsible for the release of the contents of the vesicles. The apparent discrepancy between these data and the morphological data which indicate that the bicarbonate extracted vesicles remain closed can be reconciled by assuming that bicarbonate produces a transitory opening of the vesicles. However, another possible interpretation of these data will be discussed later.

Discussion

The increased ratio of phospholipid to protein and the increased specific activity of glucose-6-phosphatase and other enzymes in the salt-extracted vesicles indicate that this fraction represents microsomal membranes in a considerably higher state of purification than microsomal membranes isolated by other procedures.

An apparent exception is M fraction, which as originally described by Ernster *et al.* (1962) is more highly enriched in phospholipid and the activity of glucose-6-phosphatase than

TABLE IV: Serum Protein Content of Microsomes during Salt and Detergent Extraction.[a]

Sample	Serum Protein Content: % of Total Microsomal Protein	Serum Protein Content: mg/g of Liver[b]
Crude microsomes	14.6	4.0
Microsomes extracted sequentially with		
0.14 M NaCl	15.5	3.1
1.0 M NaCl	21.3	3.3
0.1 M $NaHCO_3$–0.1 M Na_2CO_3	3.2	0.45
0.075% sodium deoxycholate	0	0

[a] Immunoprecipitation with antibody to rat serum was carried out as described in Methods. [b] The serum protein per gram of liver was calculated by multiplying the per cent serum protein by the yield of microsomal subfraction content per gram of liver (see Table I and Figure 4).

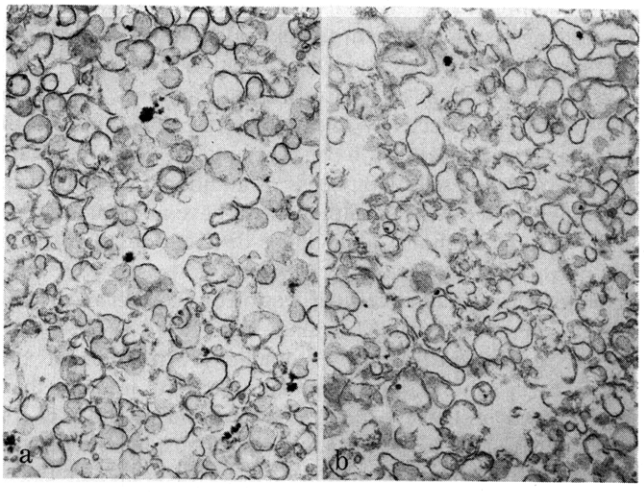

FIGURE 5: Electron micrographs of microsomal pellets extracted with sodium deoxycholate. Pellets were fixed, sectioned, and stained as described in Figure 1. (a) Salt-extracted microsomes sedimented in the presence of 0.075% sodium deoxycholate; (b) the pellets from 0.075% sodium deoxycholate sedimented in the presence of 0.3% sodium deoxycholate. Magnification 65,000×.

the salt-extracted vesicles. Studies of similar fractions in other laboratories (Redman, 1967), however, indicate levels of phospholipid which are considerably lower than originally reported but which are similar to the levels we have observed with salt-extracted vesicles. The high values may reflect the unusually high phospholipid content of the microsomes from which this fraction was first isolated: 0.6 mg of phospholipid/mg of protein compared to values between 0.3 and 0.4 usually observed for microsomes prepared in a similar manner (Dallner, 1963; Moule *et al.*, 1960). The high specific activity of glucose-6-phosphatase in the M fraction has been confirmed in other laboratories. This enzyme, however, is known to be strongly activated by concentrations of sodium deoxycholate similar to that used for the isolation of this fraction (Beaufay and de Duve, 1954). Furthermore, since the membranes appear to be partially solubilized by these intermediate concentrations of detergent, the high specific activity may also be caused by an enrichment in the M fraction of the glucose-6-phosphatase that resists solubilization by sodium deoxycholate. Preferential solubilization of certain membrane components by the detergent may also explain why the M fraction in contrast to the salt-extracted vesicles is not enriched in NADPH–cytochrome *c* reductase or cytochrome b_5 (Ernster *et al.*, 1962). These considerations illustrate the difficulty of judging the state of purity of membranes which may have been altered by the isolation procedure.

The microsomal membranes may be slightly altered by the present isolation procedure. This possibility is suggested by the slight activation of NADPH–cytochrome *c* reductase and glucose-6-phosphatase during salt extraction (indicated in Table II by the greater than 100% recoveries of these activities) and also by a slight but consistently observed change in the morphological appearance of the membranes to a more sharply defined unit membrane structure (indicated in Figure 1a–d). The activation, however, is small compared to the greater than 2-fold stimulation of these activities observed when the membranes are extensively disrupted by detergent (Beaufay and de Duve, 1954; Ernster *et al.*, 1962) and it is possible that the release of adsorbed protein, which may be present in sufficient amounts to obscure visualization of the basic membrane structure in untreated microsomes, is responsible for their altered morphological appearance. Until many additional properties have been examined, neither of these changes seem sufficient to conclude that the membranes have been extensively altered by the isolation procedure.

With the exception of relatively small quantities of protein that tend to be selectively released by low concentration of sodium deoxycholate, we have been unable to demonstrate the separation or selective release of additional protein from the salt-extracted vesicles, although we have attempted to do so by several methods. Coinciding distributions of protein and phospholipid were observed when the vesicles were subjected to density gradient centrifugation or, in experiments not shown, differential centrifugation in 0.25 M sucrose and agarose gel chromatography. Similarly, extraction of the vesicles with solutions of higher ionic strength and pH than those shown in Table I (*e.g.*, 1 M Na_2CO–1 M $NaHCO_3$ or 0.1 M Na_2CO_3) were observed to release phospholipid as well as protein from the sedimenting vesicles. These observations, as well as comparison with membranes isolated by other procedures, are consistent with the possibility that the membranes are relatively free of extraneous protein after salt extraction and to a larger extent after subsequent extraction with the dilute sodium deoxycholate.

The electron microscopic evidence that the membranes remain as intact closed vesicles during the salt-extraction procedure suggests that nearly half the total microsomal protein released by this treatment may have originally been adsorbed to the outer surface of the vesicles. Only a fraction of this protein can be accounted for by the ribosomes which constitute about 15% of the total microsomal protein (Moule *et al.*, 1960; Petermann and Hamilton, 1952). Earlier evidence that microsomes contain significant amount of hemoglobin (Paigen, 1956) and lactic dehydrogenase (Paigen and Wenner, 1962), and that these are released by salt solutions, suggests that these and probably other soluble proteins found predominantly in blood or cytoplasm may account for at least part of the adsorbed protein.

It is not clear which step or steps are responsible for the release of the contents of the vesicles. The release of serum protein suggests that most of the content protein may be released during bicarbonate extraction. However, because it has not been clearly shown that serum protein is confined exclusively to the contents of the vesicles, the possibility remains that bicarbonate removes serum protein adsorbed to the outer surface of the vesicles and that only the smaller amount released by 0.075% deoxycholate represents content protein. This possibility could explain why hypotonic shock is not a very effective method for releasing serum protein from the vesicles (Peters, 1962). It could also provide an explanation for recent findings that the maximum specific activity of newly synthesized serum albumin in isolated rough and smooth endoplasmic reticulum, which were not salt washed and therefore might contain adsorbed serum protein, is unexpectedly lower than in Golgi, which were isolated in the presence of 0.1 M phosphate (Peters *et al.*, 1971).

The indications from this study that microsomes and microsomal subfractions regarded by other workers as representing membranes are grossly contaminated by adsorbed and entrapped protein has lead to a reexamination of the question of the multiplicity of the protein components of these membranes and to the conclusion that the multiplicity is considerably less than had first seemed apparent (Hinman and Phillips, 1970). From this recent study, two findings pertinent to the present investigation have emerged. One is that the membranes from separated rough and smooth endoplasmic reticulum, after purification by the present procedure, exhibit essentially identical protein patterns during acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. This result, which differs from findings with unwashed membrane preparations (Keihn and Holland, 1970), justifies our decision to purify the membranes from total microsomes, without regard as to whether they are derived from rough or smooth endoplasmic reticulum. The second finding is that the protein compositions of smooth microsomes, which contain relatively small quantities of adsorbed protein compared to rough surfaced microsomes, remain relatively unaltered during the present purification procedure. This finding provides strong additional support for our conclusion that the compositional integrity of the membranes is not destroyed by this procedure.

Since the original observation of Rothschild (1963) that hepatic microsomal membranes segregate into several bands during density gradient centrifugation, the significance of this heterogeneity has been the subject of numerous investigations. Since the vesicles are derived from several morphologically distinct classes of intracellular membranes (*i.e.*, largely rough and smooth surfaced endoplasmic reticulum but also including small amounts of Golgi and plasma membrane) it has been assumed that these membranes are structurally heterogeneous.

On the other hand, several lines of biochemical and morphological evidence discussed in detail earlier (Manganiello and Phillips, 1965; Hinman and Phillips, 1970) suggest that much of this heterogeneity is due to factors (*e.g.*, the presence or absence of attached ribosomes and nonuniform fragmentation of the endoplasmic reticulum during cell disruption) which bear little direct relationship to the structure of the membranes and that the membranes may be considerably less heterogeneous than had first seemed apparent. Support for this interpretation is provided by the present observation that microsomal membranes possess a highly uniform buoyant density after the removal of attached ribosomes and adsorbed protein by the salt extraction procedure. The essentially quantitative recoveries of microsomal phospholipid and each of the enzymes examined rule out the alternative possibility that a single class of membranes has been selected by the isolation procedure.

The separation of the ribosomal and membranous elements of microsomes has routinely been carried out by methods in which microsomes are treated with relatively high concentrations of sodium deoxycholate and although the ribosomes are released as structurally and functionally intact particles by this treatment, it is clear that the membranes are extensively disrupted. Conditions of high ionic strength provide an alternative method for the separation of these constituents, but in this case the membranes appear to remain intact, while the ribosomes are destroyed. It is of interest in terms of the relationship between the ribosomes and the membranes to which they are attached to note that the separation of the ribosomes and membranes does not appear to have been achieved without extensive disruption of one or the other component.

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