Isolation of Intracellular Membranes by Means of Sodium Carbonate Treatment: Application to Endoplasmic Reticulum

YUKIO FUJIKI, ANN L. HUBBARD, STANLEY FOWLER, and PAUL B. LAZAROW
The Rockefeller University, New York 10021; and Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT A rapid and simple method for the isolation of membranes from subcellular organelles is described. The procedure consists of diluting the organelles in ice-cold 100 mM Na_2CO_3 followed by centrifugation to pellet the membranes. Closed vesicles are converted to open membrane sheets, and content proteins and peripheral membrane proteins are released in soluble form. Here we document the method by applying it to various subfractions of a rat liver microsomal fraction, prepared by continuous density gradient centrifugation according to Beaufay et al. (1974, *J. Cell Biol.* 61:213–231). The results confirm and extend those of previous investigators on the distribution of enzymes and proteins among the membranes of the smooth and rough endoplasmic reticulum. In the accompanying paper (1982, *J. Cell Biol.* 93:103–110) the procedure is applied to peroxisomes and mitochondria.

Because membranes play a variety of vital roles in the functioning of cells, an understanding of the properties of membranes is important in many areas of biology. Procedures have been devised for the isolation of plasma and intracellular membranes from many sources (cf. reference 1), but, to date, only the architecture of the red blood cell membrane is understood in any detail (2).

Biochemical investigations of rat liver endoplasmic reticulum generally use a microsomal fraction that is heterogeneous in two respects. First, the microsomal vesicles are derived not only from the endoplasmic reticulum (ER) but also from the Golgi apparatus, the outer mitochondrial membrane, the endocytic compartment, and the plasma membranes of hepatocytes, Kupffer cells, and endothelial cells. Second, the vesicles that are derived from the ER possess three distinct parts: the membranes themselves, the attached ribosomes, and the lumenal contents. Beaufay, Amar-Costesec, and colleagues (3, 4) investigated the first source of heterogeneity by subfractionating the microsomal fraction after a variety of experimental manipulations; they recognized six groups of enzymes in the fraction and identified the intracellular origin of each. Adelman et al. (5), Kreibich et al. (6), and Kreibich and Sabatini (7) investigated the compartmental heterogeneity of rough microsomes by dissecting them with puromycin, salt, and low concentrations of detergents and were able to distinguish contents from membrane components and ribosomal proteins.

Here we describe a simple, one-step procedure for the isolation of membranes from organelles, and demonstrate its application to various microsomal subfractions. In addition, we compare the polypeptide compositions of highly purified rough and smooth microsomes prepared according to Beaufay et al. (3). In the accompanying paper (8) we use this procedure to compare the integral membrane proteins of peroxisomes, ER, and mitochondria. An abstract of the method has appeared (9).

MATERIALS AND METHODS

Preparation and Subfractionation of Microsomes

Microsomes were prepared in conjunction with the isolation of peroxisomes. A "psi" fraction (consisting of microsomes and cytosolic proteins, and equivalent to the P + S fraction of de Duve et al. [10]) was prepared by differential centrifugation of homogenates of livers of female Sprague-Dawley rats treated with Triton WR-1339 (11). This fraction was centrifuged for 40 min at 50,000 rpm in the Beckman 50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The pellet (total microsomes or P fraction) was resuspended in 250 mM sucrose containing 0.1% ethanol and subfractionated by means of isopycnic centrifugation in a linear density gradient of sucrose according to Beaufay et al. (3), except that the density gradient extended from 1.10 to 1.27 g/cm^3 and the sucrose solutions contained 5% Dextran 10 and 0.1% ethanol (11). Polysomes were prepared from rat liver according to Taylor and Schimke (12).

Removal of Ribosomes

Two established procedures were used to remove ribosomes from rough

microsomes. Puromycin-KCl treatment (5): The rough microsomes were incubated with 1 mM puromycin in 500 mM KCl, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, for 10 min at 37°C, and then centrifuged for 30 min at 39,000 rpm in a 50 Ti rotor. Pyrophosphate treatment (4): The rough microsomes were suspended in 250 mM sucrose containing 15 mM sodium pyrophosphate at pH 8.3 and centrifuged for 2 h at 50,000 rpm in the 50 Ti rotor. After each treatment, the pellets were washed in 250 mM sucrose by resuspension and centrifugation.

Isolation of Membranes—Carbonate Procedure

Microsomal fractions were diluted 50- to 1,000-fold with 100 mM sodium carbonate, pH 11.5, to bring the protein concentration to 0.02 to 1 mg/ml, and incubated at 0°C for 30 min. The suspensions were centrifuged at 0–4°C for 1 h at 50,000 rpm in polycarbonate tubes in the Beckman 50 Ti rotor (~233,000 g_{max}). The supernatants were decanted and the membrane pellets were gently rinsed once with ice-cold distilled water. The pellets were either dissolved in 500 mM NaOH for protein determinations, or extracted with chloroform/methanol for phospholipid analyses, or dissolved in SDS for PAGE.

Analytical Methods

Protein was determined by the method of Lowry et al. (13) using bovine serum albumin as standard. Esterase (14), cytochrome b_5 (14), RNA (15), NADPH-cytochrome *c* reductase (6), and NADH-ferricyanide reductase (16) were assayed as described. Phospholipids were quantitatively extracted by the method of Bligh and Dyer (17). They were ashed and total phosphate was measured according to Ames and Dubin (18).

SDS PAGE

SDS PAGE was carried out as described previously (19), except that 27 mM iodoacetamide was used for alkylation. Membrane pellets were dissolved directly in SDS sample buffer. Dilute proteins in Na_2CO_3 supernatants were first precipitated with 10% trichloroacetic acid and washed twice with ice-cold ethyl ether to remove residual trichloroacetic acid.

Morphological Analysis

The membrane pellets, suspended in 250 mM sucrose, were fixed in 2% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, for 30 min at 0°C and then centrifuged at 45,000 rpm for 30 min. The pellet was postfixed in 1% OsO_4. The samples were treated with 0.5% uranyl acetate (20), dehydrated, and embedded in Epon. In some experiments, membrane pellets prepared by the carbonate procedure were fixed as such, without resuspension. Membrane thickness was measured with a Bausch & Lomb measuring magnifier with internal scale (0.1-mm divisions; E. F. Fullam, Inc., Schenectady, NY) on prints with final magnification of 133,000.

Materials

Sodium carbonate (certified ACS Grade) was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Puromycin dihydrochloride was obtained from ICN Nutritional Biochemicals (Cleveland, OH). SDS was purchased from BDH Chemicals, Ltd. (Poole, England). Molecular weight standard protein kit and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade. Rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

RESULTS

Properties of Microsomal Subfractions

Total microsomes (P fraction) were subfractionated by isopycnic centrifugation in a sucrose gradient (Fig. 1). Cytochrome b_5 and esterase, two biochemical markers for the membranes of ER-derived vesicles, were most concentrated in fractions 8 and 9, respectively, at densities of ~1.15–1.16; their concentrations decreased gradually with increasing density. RNA, a main constituent of ribosomes, was most concentrated near the bottom of the gradient, but was present in appreciable quantities at densities as low as 1.12. These three markers were selected because they belong to the three classes of microsomal constituents identified by Beaufay et al. (3). Their distributions confirm the results of these authors that smooth and rough

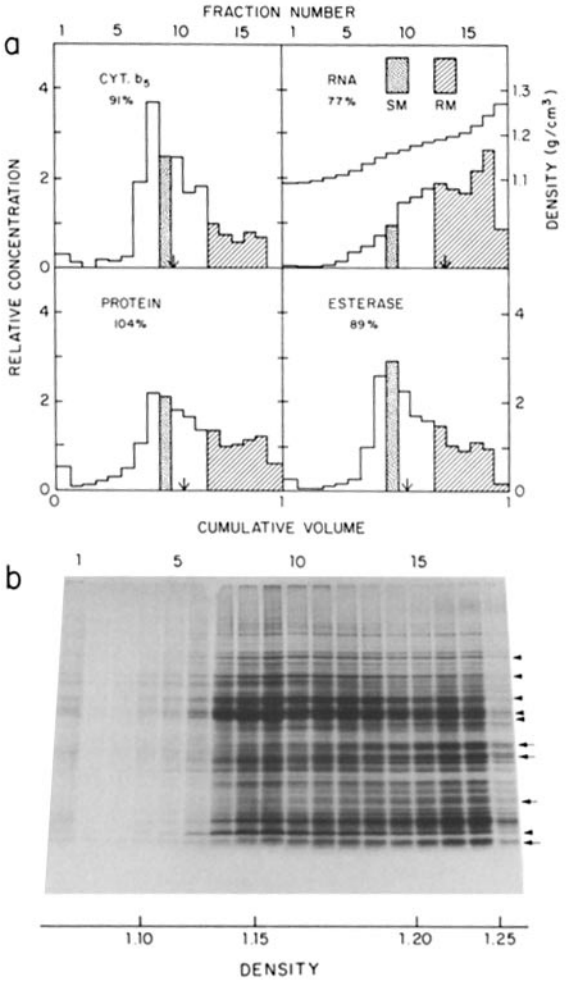


FIGURE 1 Subfractionation of microsomes by equilibrium density centrifugation. (*a*) Distribution of protein, esterase, cytochrome b_5, and ribonucleic acid. Percentages give recovery of each constituent, arrows indicate the median densities. Results were plotted acccording to Beaufay et al. (29). Measured densities of fractions are included at upper right. The smaller peak of protein at density 1.24 represents microsomes that have accumulated on the dense cushion of sucrose at the bottom of the gradient. The larger amount of RNA there indicates that these microsomes bear many ribosomes, as would be expected. (*b*) SDS PAGE analysis. 6 μl of each fraction (containing 10–220 μg of protein) was loaded onto the gel.

microsomes cannot be completely separated on the basis of density, and that there are two groups of membrane markers with slightly, but significantly, different distributions.

The distribution of microsomal polypeptides in the gradient, as analyzed by SDS PAGE (Fig. 1 *b*), exhibited two obvious patterns. Some, marked by arrowheads, tended to follow the esterase distribution and are likely to be membrane proteins. Others (arrows) followed the RNA distribution and are probably ribosomal proteins.

Fractions with a density of 1.19 or more were pooled into a "rough microsomal fraction" or "RM" (shaded area in Fig. 1 *a*). As shown in Fig. 2 *a*, it consisted almost exclusively of closed vesicles heavily studded with ribosomes.

The microsomal fraction of density 1.15 (fraction 9, Fig. 1 *a*) was taken as the "smooth microsomal fraction" or "SM." It consisted predominantly of sealed smooth vesicles, mainly derived from the smooth ER (not illustrated). Lighter fractions were avoided because they are richer in Golgi complex ele-

TABLE I

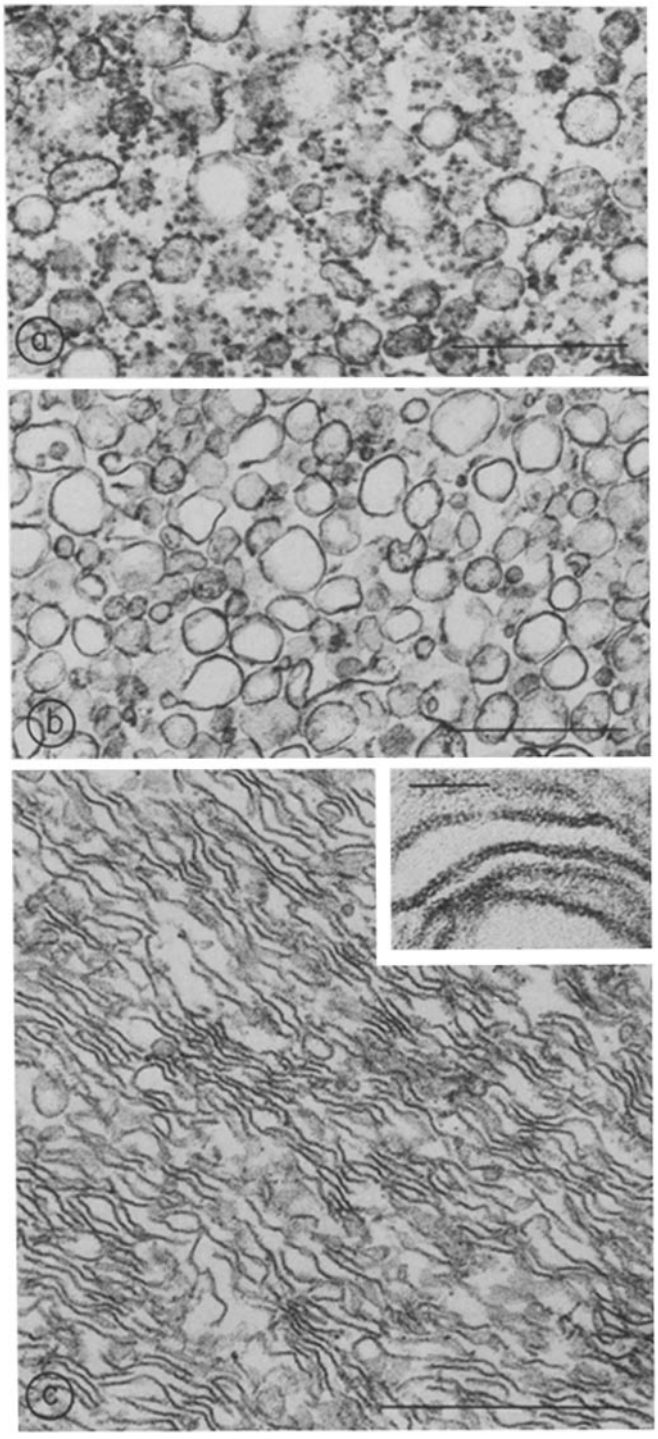
Subfractionation of Microsomes by Isopycnic Centrifugation *

	Homogenate activity	Microsomes specific activity	Entire gradient		Microsomal subfractions		
			Median density	Recovery	Rough	Smooth	RM/SM‡
	U or mg§/g liver	*U or mg/mg protein*	*g/cm³*	*%*	*U or mg/mg protein*		
Protein	229 ± 21		1.1687 ± 0.0010	109 ± 9			
Esterase	215 ± 54	5.04 ± 1.26	1.1625 ± 0.0050	90 ± 5	2.51 ± 0.67	6.79 ± 3.22	0.43 ± 0.21
Cytochrome b_5		3.65 ± 1.57	1.1571 ± 0.0065	85 ± 11	0.99 ± 0.33	5.75 ± 4.32	0.25 ± 0.21
RNA	13.5	0.209 ± 0.113	1.1942 ± 0.0055	86 ± 10	0.29 ± 0.15	0.073 ± 0.032	3.83 ± 0.54

* Complementary data to Fig. 1. Means and standard deviations of three experiments.

‡ Ratios for individual experiments varied, depending on which fractions were chosen as "RM" and "SM." The mean ratios differ somewhat from the ratio of the means of the two preceding columns.

§ μmol/min for esterase; mg for protein, cytochrome b_5, and RNA.



ments and fragments of the plasma membrane and outer mitochondrial membrane (3). The ratio of cytochrome b_5 to RNA was 23-fold greater in the SM than in the RM. Other quantitative data (Table I) were generally in agreement with previous reports (3, 21, 22).

Removal of Ribosomes from Rough Microsomes

In preliminary experiments, pyrophosphate treatment (4) was more effective than puromycin plus a high concentration of salt (5) in removing ribosomes from rough microsomes, as judged by electron microscopy and by SDS PAGE. Pyrophosphate converted vesicles heavily studded with ribosomes (Fig. 2*a*) to smooth, but still sealed, vesicles (Fig. 2*b*). SDS PAGE analysis showed that the polypeptide composition of stripped rough microsomes (Fig. 3, panel *I*, lane *A*) resembled that of smooth microsomes (Fig. 1*b*, fraction 9) or stripped smooth microsomes (Fig. 3, panel *II*, lane *A*), consistent with the absence of ribosomes. Differences between stripped rough and smooth microsomes include the presence of the ribophorins (23) in the former (Fig. 3).

Carbonate Treatment

The carbonate procedure was applied to stripped rough microsomes. As observed by electron microscopy, it converted the closed vesicles of Fig. 2*b* to open sheets of membranes ranging from 0.1 to 0.5 μm or more in size (Fig. 2*c*). As revealed by SDS PAGE analysis, the carbonate released a number of proteins in soluble form (Fig. 3, panel *I*). However, most of the microsomal polypeptides were recovered with the membranes. The sum of the patterns of the membranes and soluble proteins agrees closely with that of the starting material, indicating that the carbonate procedure was nondestructive. Very similar SDS PAGE results were obtained when the SM (that had also been subjected to pyrophosphate treatment to remove residual ribosomes) was treated with carbonate (Fig. 3, panel *II*).

We found that it was not necessary to remove the ribosomes before carbonate treatment to obtain clean membranes. Carbonate simultaneously removed ribosomes and produced open membrane sheets indistinguishable from those of Fig. 2*c*. As revealed by SDS PAGE analysis (Fig. 4, lane *A*), the polypeptides in the resulting membranes were nearly identical to those

FIGURE 2 Electron microscope analyses. (*a*) Rough microsomes. (*b*) Rough microsomes after removal of ribosomes by pyrophosphate treatment. (*c*) Rough microsomes after pyrophosphate and then carbonate treatment. All micrographs are of the middle of the pellets. Bars, 0.5 μm. (*a* and *b*) × 48,000. (*c*) × 65,000. *Inset* in *c* shows trilaminar structure of the membrane. Bar, 50 nm. × 212,000.

in membranes purified by sequential pyrophosphate and carbonate treatment (Fig. 4, lane *B*). There were only a few minor differences in intensity. Moreover, the carbonate method was applied to total microsomes with similar results (Fig. 4, lane *D*).

Characteristics of Carbonate-treated Microsomes

Morphologically, membranes prepared by this procedure retained a trilaminar appearance (Fig. 2*c*, *inset*). According to SDS PAGE analysis they also retained integral membrane proteins characteristic of the ER, including cytochromes P-450 and b_5 in both smooth and rough microsomes, and the ribophorins in rough microsomes (Fig. 3). Phospholipid, a measure of the membrane bilayer, was retained almost quantitatively in the carbonate-treated microsomes (Table II). Finally, two integral membrane enzymes of RM and SM, NADH-ferricyanide reductase and NADPH-cytochrome *c* reductase, remained associated with the membranes after carbonate treatment and were active (Table III). However, they could be solubilized by a combination of Triton X-100 and urea. The percentage of protein recovered in the membranes varies with the load of ribosomes, as expected. After removal of the ribosomes, 81% of the protein of both smooth and rough microsomes was recovered with the membranes (Table II). The membranes had a thickness of 6.1 ± 0.6 nm (mean ± standard deviation) after carbonate treatment, compared with 6.3 ± 0.4 before.

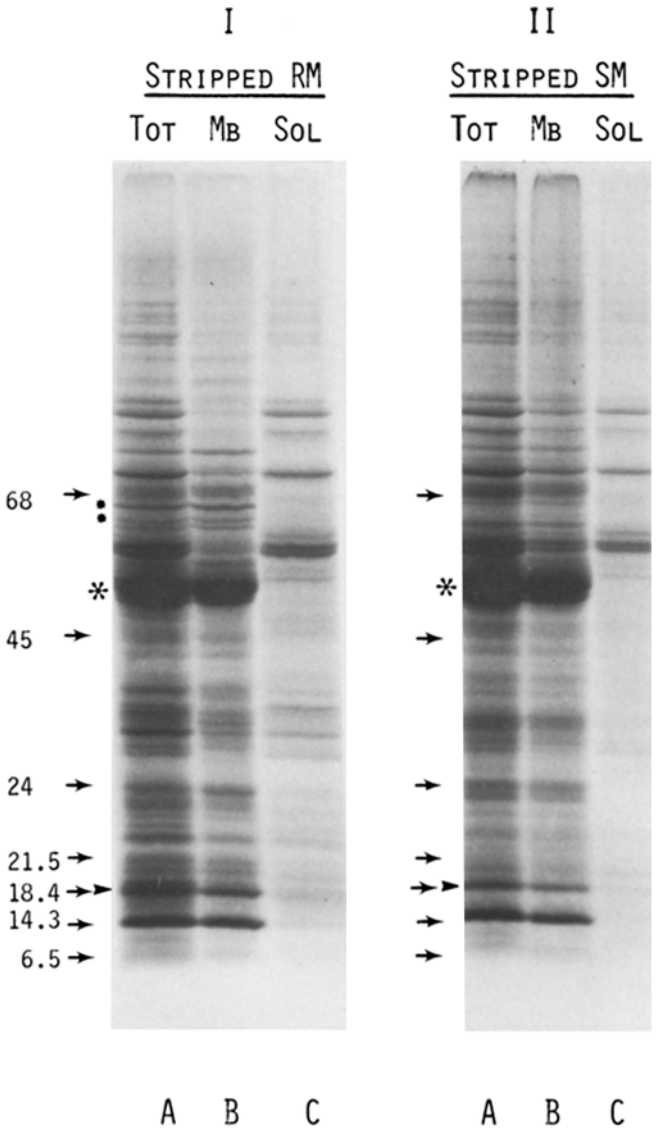


FIGURE 3 Sodium carbonate treatment of microsomal subfractions; analysis by SDS PAGE. Panel *I*: (*A*) Total proteins, (*B*) membrane proteins, and (*C*) soluble proteins derived from 200 μg of stripped rough microsomes. Panel *II*: Products of carbonate treatment of 200 μg of stripped smooth microsomes. The ribophorins (small asterisks) and cytochrome P-450 (large asterisk) were identified by comparing these SDS PAGE results with those of Kreibich et al. (30). Cytochrome b_5 (arrowhead) was identified by its similar mobility to purified bovine liver cytochrome b_5 (generously supplied by P. Strittmatter (University of Connecticut), not illustrated) and by comparison with the results of Rachubinski et al. (31). Arrows indicate the positions of the protein standards; molecular masses given in kilodaltons: bovine serum albumin (68), ovalbumin (45), trypsinogen (24), soybean trypsin inhibitor (21.5), β-lactoglobulin (18.4), lysozyme (14.3), and bovine lung trypsin inhibitor (aprotinin) (6.5).

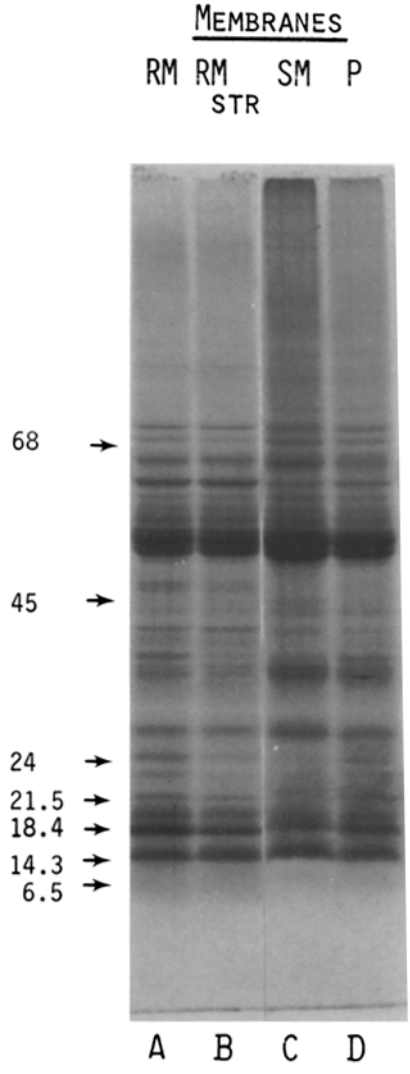


FIGURE 4 Comparison by SDS PAGE of membranes prepared from various microsomal subfractions by the sodium carbonate procedure. 80–90 μg of membranes from: (*A*) native rough microsomes; (*B*) stripped rough microsomes; (*C*) native smooth microsomes; and (*D*) total microsomes. Standards as in Fig. 3.

Release of Content Proteins

To assess the extent to which lumenal proteins were released by the carbonate procedure, a rat was pulse-labeled for 8 min with [^{3}H]leucine (Table III). Under these conditions, a large part of the incorporated label is found in secretory proteins within the cisternae of the ER, although label is also found in cell sap proteins, and organelle proteins including the ER membranes (6, 24). As shown in Table III, 79% of the radioactivity in rough microsomes was released by the carbonate treatment. This suggests that the bulk of the microsomal contents were released by the carbonate procedure, corroborating the SDS PAGE results shown in Fig. 3.

Requirements of the Carbonate Procedure

Several experiments were carried out to determine the essential feature(s) of the 100 mM Na_2CO_3 procedure. Aliquots of stripped rough microsomes were adjusted to pH 6, 7, 8, 9, 10, 11, or 12 with 1 mM Tris/HCl or 1 mM $K_2B_4O_7$. After treatment at pH 11, the pattern of membrane polypeptides obtained was similar to that resulting from carbonate treatment. pHs less than 11 gave unsatisfactory results (not illustrated). 100 mM K_2CO_3 replaced 100 mM Na_2CO_3, but 250 mM $NaHCO_3$ or NaCl or KCl were ineffective. Thus pH rather than ionic strength appeared to be crucial. A second application of Na_2CO_3 to isolated membranes did not alter the polypeptide composition of the membrane. Also, the same pattern of polypeptides was observed when the isolation of membranes was carried out in the presence of a mixture of protease inhibitors (chymostatin, leupeptin, pepstatin, antipain, soybean trypsin inhibitor, bovine trypsin inhibitor, and phenylmethylsulfonylfluoride) (not illustrated). Therefore proteolytic degradation appeared not to be a problem.

Several other methods known to remove peripheral membrane proteins were carried out on stripped rough microsomes: 90 mM NaOH at 0°C for 15 min (25), 5 mM EDTA (pH 7.4) at room temperature for 1 h (26), and 1 mM *p*-chloromercuribenzoate in sodium phosphate buffer (pH 8) at 0°C for 30 min (25), in each case followed by centrifugation at 50,000 rpm for 1 h. SDS PAGE analysis of the pellets revealed patterns of polypeptides generally similar to that of Fig 3, lane *B*. However, some additional polypeptides were found in the pellets from each of these three procedures that were absent from the membranes prepared with Na_2CO_3, possibly because some of these procedures may not unseal closed vesicles. Thus the carbonate procedure appears to be the most effective of those tested.

TABLE II

Application of Carbonate Procedure to Various Membranes

	Protein in membranes			Phospholipid in membranes		
	n	Mean	Range	*n*	Mean	Range
		% of membrane and soluble			% of membrane and soluble	
Total microsomes	2	53	±5			
Rough microsomes	2	41	±7			
Stripped rough microsomes	3	81	±15*	2	94	±3
Smooth microsomes	2	57	±5	1	96	
Stripped smooth microsomes	1	81				

* Standard deviation.

DISCUSSION

Treatment with 100 mM Na_2CO_3 at O°C has been shown to remove ribosomes from ER membranes, to convert sealed vesicles into flat membrane sheets, and to effectively release cisternal contents. The membranes, recovered in highly purified form by centrifugation, appear to be intact by several criteria: they retain integral membrane proteins (cytochrome b_5, cytochrome P-450, and ribophorins I and II); they retain integral membrane enzymes (NADPH-cytochrome *c* reductase and NADH-ferricyanide reductase) in active form; they retain phospholipids and they exhibit a normal trilaminar appearance.

The carbonate procedure appears to be at least as effective as the established low detergent procedure of Kreibich et al. (6, 7) in selectively releasing microsomal contents. The principal polypeptides released by carbonate (as analyzed by SDS PAGE) are similar to those solubilized by low detergent concentrations or by sonication (7). Moreover, carbonate releases 79% of pulse-labeled proteins, in comparison to the 40–60% released by low detergent concentrations under various conditions. The carbonate procedure solubilizes ~20% of the total protein of stripped rough or smooth microsomes, which is comparable to or slightly greater than the proportion released by the detergent method.

Scheele et al. (27) reported that some basic proteins may contaminate rough microsomes by virtue of an ionic adsorption

TABLE III

Distribution of Radioactivity and Enzymes after Various Treatments of Rough Microsomes *

	Radioactivity		NADH-Ferricyanide reductase		NADPH-Cytochrome *c* reductase	
	Supernatant	Recovery‡	Supernatant	Recovery	Supernatant	Recovery
	% of pellet and sup	%	% of pellet and sup	%	% of pellet and sup	%
Control§	6	70	1	83	1	112
Triton-Urea¶	93	90	94	136	96	80
Carbonate**	79	91	4	91	7	81

* Rats fasted for 18 h received 5 μCi [^{3}H]leucine/g body weight by injection into the portal vein under ether anesthesia and were sacrificed 8 to 10 min later. Rough microsomes were prepared from the liver according to Adelman et al. (5). Aliquots containing 1 mg of protein were treated for 30 min at 4°C and then centrifuged for 30 min at 35,000 rpm. The pellets were resuspended in 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$ (STKM). *sup*, supernatant.

‡ Recovery = (pellet & supernatant)/starting material × 100%.

§ STKM, see footnote.*

¶ 2% Triton X-100, 2 M urea, 50 mM Tris-HCl (pH 8.0).

** 100 mM Na_2CO_3.

onto ribosomes. The carbonate procedure would remove these together with the ribosomes.

Singer and Nicolson (28) have distinguished between integral and peripheral membrane proteins. The latter are defined by three criteria: (*a*) they are released by "mild treatments, such as an increase in the ionic strength of the medium or the addition of a chelating agent;" (*b*) "they dissociate free of lipids;" and (*c*) "they are relatively soluble in neutral aqueous buffers." More recently, the definition of peripheral membrane proteins has been broadened to include proteins released by stronger reagents, including protein denaturants (e.g., 6 M guanidine hydrochloride or 0.1 M NaOH) that dissociate some of the membrane proteins, while leaving the remainder associated with all the phospholipid (see review by Steck [2]). In contrast, integral membrane proteins are amphipathic and are only solubilized by conditions that disrupt the lipid bilayer. It appears from the evidence that we present here that the carbonate procedure discriminates between peripheral and integral membrane proteins. Its mechanism of action was found to depend primarily on pH, and the pH employed, 11.5, is close to the pH of 12 demonstrated by Steck and Yu (25) to effectively remove peripheral membrane proteins from the red blood cell membrane. In fact, the carbonate procedure appears to be even more effective with microsomes than the other procedures examined.

The carbonate procedure has several advantages over other methods discussed above. First, it is rapid, simple, and in our experience, highly reproducible. The conditions of its use are not critical, in contrast to the low detergent concentration method, which is dependent on protein concentration and ionic strength (6, 7). Due to the buffering capacity of the carbonate, control of the pH is easier than with dilute alkali.

As described in the accompanying paper (8), we have successfully applied the carbonate procedure to other intracellular membranes of rat liver, those of the peroxisomes and mitochondria. In addition, the method has proved useful for studying the integral membrane proteins of rat liver plasma membranes (A. L. Hubbard and A. Ma, manuscript in preparation).

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