

# ON THE SPATIAL ARRANGEMENT OF PROTEINS IN MICROSOMAL MEMBRANES FROM RAT LIVER

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## ABSTRACT

Rat liver rough microsomes were labeled enzymatically with  $^{125}\text{I}$  using lactoperoxidase and glucose oxidase. In intact microsomes only proteins exposed on the outside face of the microsomal membrane were iodinated. Low concentrations of detergent (0.049% deoxycholate) were used to allow entrance of the iodination system into the vesicles without disassembling the membranes. This led to iodination of the soluble content proteins and to an increased labeling of the membrane proteins.

The distribution of radioactivity in microsomal proteins was analyzed after separation by sodium dodecyl sulfate acrylamide gel electrophoresis. Most membrane proteins were labeled when intact microsomes were iodinated. No major membrane proteins were exclusively labeled in the presence of low detergent concentrations or after complete membrane disassembly. Therefore it is unlikely that there are major membrane proteins, other than glycoproteins, present only on the inner membrane face or completely embedded within the microsomal membrane.

Microsomal proteins were also labeled by incubating rough microsomes with  $^3\text{H}$ - $\text{NaBH}_4$  after reaction with pyridoxal phosphate. Microsomal membranes were permeable to these small molecular weight reagents as shown by the fact that proteins in the vesicular cavity as well as membrane proteins were labeled with this system.

## INTRODUCTION

In recent years a number of procedures have been introduced to label and specifically analyze proteins exposed on one side of cellular membranes (for reviews see Wallach, 1972; Steck and Fox, 1972). Appropriate labeling systems were added to membrane-enclosed structures such as erythrocytes (Carraway et al., 1971; Phillips and Morrison, 1971; Hubbard and Cohn, 1972; Segrest et al., 1973), platelets (Nachman et al., 1973), intact cells (Baur et al., 1971; Marchalonis et al., 1971; Poduslo et al., 1971; Kinzel and Mueller, 1973), virus particles (Stanley and Haslam, 1971; Rifkin

et al., 1972), or isolated cell organelles (Welton and Aust, 1972). The present study was carried out with rat liver rough microsomes (RM) which are closed vesicles derived from the rough endoplasmic reticulum (ER) during cell fractionation. These microsomes retain the normal membrane orientation of the ER cisternae with ribosomes bound only to their outer face and the luminal content preserved in the vesicular cavity. We have recently shown (Kreibich et al., 1973 *a*) that low detergent concentrations can be used to extract the content of microsomes without disassembling

the membranes. Secretory products such as serum albumin and other proteins with high rates of amino acid incorporation are released from the vesicular cavities by low detergent concentrations, but phospholipids, ribosomes, and other typical components of the ER membranes such as cytochrome *b<sub>5</sub>*, NADH- and NADPH-cytochrome *c* reductases remain bound to sedimentable vesicles.

An extensive study led us to the conclusion that levels of detergent below the critical micellar concentration cause a reversible structural change in the microsomal membranes which allows escape of content and penetration of macromolecules such as polysaccharides and proteases into the vesicular cavities (Kreibich et al., 1973 *a*).

In the experiments described here, two systems have been used to label microsomal proteins. A lactoperoxidase (LPO) catalyzed iodination (<sup>125</sup>I) (Phillips and Morrison, 1970, 1971; Marchalonis, 1969) was carried out using glucose and glucose oxidase (GO) to generate low levels of H<sub>2</sub>O<sub>2</sub> (Hubbard and Cohn, 1972). Since LPO is thought to participate directly in the iodination reaction (e.g., Bayse et al., 1972 *a, b*), <sup>125</sup>I labeling should normally occur in the outer microsomal face. Proteins contained in the vesicular cavity or attached to the inside membrane face should be unlabeled, unless low detergent concentrations are present during labeling.

Microsomes were also labeled with pyridoxal phosphate and tritiated sodium borohydride (Rifkin et al., 1972). For microsomes it has been proposed that small, charged molecules such as pyridoxal phosphate cannot penetrate the vesicular membrane (Nilsson et al., 1971, 1973).

#### MATERIALS AND METHODS

Rat liver RM prepared as described previously (Adelman et al., 1973) were washed by centrifugation (15 min at 17,000 *g*) in a high salt buffer (HSB; 50 mM Tris-HCl, pH 7.5, 500 mM KCl, 5 mM MgCl<sub>2</sub>) and resuspended (3 mg protein/ml) in a low salt buffer (LSB; 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>) containing 10 mM glucose. The permeability of the membranes was altered by adding deoxycholate (DOC) to a final concentration of 0.049%, or membranes were dissolved by raising the DOC concentration to 0.39 or 0.78% (Kreibich et al., 1973 *a*). The iodination was carried out at 3°C under basically the same conditions used by Hubbard and Cohn (1972). The incubation mixture contained per milliliter 5–10 μg of LPO (B grade, Calbiochem, La Jolla, Calif.), approximately 0.5 μg GO (type V, Sigma Chemical Co., St. Louis, Mo.)

and 50–100 μCi of carrier-free [<sup>125</sup>I]NaI (New England Nuclear, Boston, Mass.). The reaction was stopped after 10 min by diluting 20 times with LSB containing 10<sup>-5</sup> M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

Samples containing low concentrations of DOC (<0.049%) or no detergent were underlaid with ~8 ml of 20% sucrose containing LSB and centrifuged for 60 min at 60,000 *g*. After the surfaces were rinsed, the pellets were resuspended to the original volume in H<sub>2</sub>O or LSB. The supernatants of samples with 0.049% DOC and microsomes dissolved with high detergent concentrations (0.392 and 0.784% DOC) received cold TCA to a final concentration of 5%. The coprecipitate of DOC and protein was washed and redissolved by adding 0.1 N NaOH to neutral pH.

Labeling of microsomal proteins with pyridoxal phosphate and subsequent reduction with [<sup>3</sup>H]-NaBH<sub>4</sub> were carried out essentially as described by Rifkin et al. (1972). Freshly prepared RM were washed twice (15 min at 17,000 *g*) in HSB containing HEPES (Sigma Chemical Co., St. Louis, Mo.) instead of Tris and resuspended (3 mg protein/ml) in LSB (HEPES was again exchanged for Tris). Aliquots of the suspension with or without DOC (see above) were incubated for 30 min with 0.2 or 1.0 mM pyridoxal phosphate (Calbiochem, La Jolla, Calif.). After incubation at 37° or 0°C, respectively, samples were cooled to 0°C and treated with fivefold molar excess of [<sup>3</sup>H]NaBH<sub>4</sub> (Amersham/Searle Corp., Arlington Heights, Ill., sp act of 5–7.2 Ci/mmol) which was dissolved just before use in a small amount of 10 mM KOH. Membranes and supernatants were separated and treated as described for the iodination.

Sucrose density gradient analysis, digestion of radioactively labeled microsomes with trypsin and chymotrypsin (Kreibich et al., 1973 *a*), and discontinuous sodium dodecyl sulfate (SDS) acrylamide gel electrophoretic analysis of RM and of RM subfractions were carried out by procedures to be described.<sup>1</sup> Gels were 7 or 10% in acrylamide; the spacer gel was in all cases 3%; the ratio of acrylamide to bisacrylamide was 30 to 0.8 and all samples were reduced with mercaptoethanol before loading. For determination of radioactivity in iodinated samples, aliquots (50–100 μl) were spotted on glass fiber filters (GF/C, Whatman, England) and processed as described for RNA precipitation (Mans and Novelli, 1961) using cold TCA which contained 10<sup>-3</sup> M NaI. Dried filters and slices from acrylamide gels were counted in a Nuclear-Chicago gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with a counting efficiency of

<sup>1</sup> G. Kreibich and D. D. Sabatini. 1973. Selective release of content from microsomal vesicles without membrane disassembly. II. Electrophoretic and immunological characterization of microsomal subfractions. Submitted for publication.

~70%. Hot TCA-insoluble tritium radioactivity was counted in aliquots processed in filter paper disks (Mans and Novelli, 1961); gel slices were dissolved with 0.5 ml of 20% H<sub>2</sub>O<sub>2</sub> and counted in a butylphenylbiphenyloxazole (PBD)-naphthalene scintillation fluid (8 g butyl-PBD, Eastman Organic Chemicals Div., Rochester, N. Y., and 80 g naphthalene/liter dioxane). The procedures to label different constituents of RM *in vivo* are described in detail in a previous paper (Kreibich et al., 1973 a).

## RESULTS

### Enzymatic Iodination of Microsomes

Intact RM were iodinated for 10 min with <sup>125</sup>I in a LPO-GO system, sedimented, and resuspended in LSB. The stability of the association of the iodinated proteins with other microsomal components was tested at detergent concentrations ranging from 0.003 to 0.39% DOC (Kreibich et al., 1973 a). The distribution of <sup>125</sup>I-labeled proteins was measured in sedimentable and soluble microsomal subfractions separated by density gradient centrifugation. Fig. 1 summarizes the detergent-dependent release of <sup>125</sup>I-labeled proteins from the membranes and compares it with that of other components of RM labeled *in vivo* by various procedures. Throughout the whole range of DOC concentrations tested, the release of <sup>125</sup>I-labeled microsomal proteins closely followed that of membrane structural constituents such as phospholipids and membrane proteins. <sup>125</sup>I-labeled proteins were extensively released at DOC concentrations higher than 0.098% which also led to the detachment of membrane-bound ribosomes. Proteins and glycoproteins which could be labeled in a short time *in vivo* (30 min) were released at considerably lower DOC concentrations (0.025%). The results in Fig. 1 suggest that the LPO-GO system only labeled membrane constituents and not the rapidly turning over components of the vesicular content (Campbell et al., 1960; Peters et al., 1971). This would be expected if iodination depended strictly on the action of LPO, a protein of molecular weight 84,000 daltons which is unlikely to cross ER membranes. When LPO was left out of the iodination mixture very low levels of iodination were obtained (Table I). Addition of detergent (low or high levels) to the iodination mixture without LPO did not increase the low background of nonenzymatic iodination (Table I). Analysis of total microsomes in SDS discontinuous acrylamide gels revealed that the material

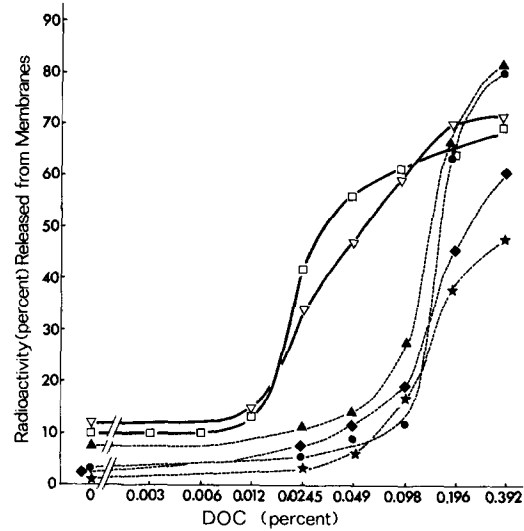


FIGURE 1 Differential effect of DOC on the release of labeled microsomal constituents in a solution of low ionic strength (LSB). RM containing different labels incorporated *in vivo* or <sup>125</sup>I incorporated *in vitro* were resuspended in LSB (3 mg protein/ml) and treated with DOC at concentrations ranging from 0.003 to 0.392% final. 0.5-ml aliquots were layered onto 10–60% sucrose density gradients containing LSB which were centrifuged for 3 h at 200,000 *g* and 3°C. For each label the percentage of the total radioactivity which was released from the membrane band and was found in the top five fractions of the gradients is plotted. □—□, rapidly labeled proteins (<sup>3</sup>H]leucine, 30 min); ▽—▽, rapidly labeled glycoproteins (<sup>3</sup>H]glucosamine, 30 min). Membrane constituents: ●—●, phospholipids (<sup>3</sup>H]choline, 4 h), ◆—◆, membrane proteins (<sup>14</sup>C]leucine for 3 days); ▲—▲, ribosomes (<sup>3</sup>H]orotic acid, 40 h); ★—★, membrane proteins labeled *in vitro* with <sup>125</sup>I for 10 min. For details on *in vivo* labeling procedures see Table I in reference (Kreibich et al., 1973 a).

iodinated in the absence of peroxidase migrated with the dye front (Fig. 2) and therefore was of low molecular weight.

When intact microsomes were iodinated with the complete LPO-GO system, sedimented, and subfractions were obtained by the low DOC treatment, SDS acrylamide gel analysis revealed that the proteins contained in the vesicular cavities were not labeled and thus had been protected from the LPO catalyzed iodination (Fig. 3, upper panel). However, the membranes recovered in the sedimentable fraction were highly labeled and the radioactivity distribution coincided qualitatively with the intensity of the Coomassie blue-stained

bands (Fig. 3). A peak of radioactivity was found (Fig. 3) at the dye front (fraction no. 100) and a smaller one ahead of it. Major radioactivity peaks were associated with proteins of mobility corresponding to 80,000, 50,000, and less than 30,000

TABLE I  
Specific Activity of RM and of Microsomal Subfractions Labeled with  $^{125}\text{I}$

	% DOC*	Total protein mg	Specific activity $10^4 \times \text{cpm}/\text{mg protein}$
Total RM (no LPO)	0	3.01	0.02
Total RM (no LPO)	0.049	3.03	0.02
Total RM (no LPO)	0.78	3.02	0.03
Supernatant ‡	0	0.09	0.04
Sediment ‡	0	2.85	0.60
Supernatant	0.049	0.12	4.20
Sediment	0.049	2.92	0.95
Total RM	0.78	3.13	2.01

\* Present during labeling.

‡ RM were recovered after labeling and fractionated with 0.049% DOC (see Materials and Methods).

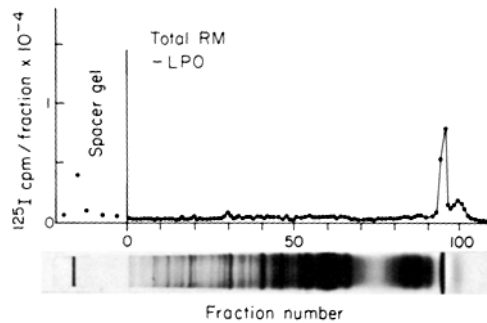


FIGURE 2 Distribution of  $^{125}\text{I}$  radioactivity in control RM incubated without LPO. RM were incubated with  $^{125}\text{I}$  as described in Materials and Methods omitting LPO. An aliquot ( $420 \mu\text{g}$  protein) was solubilized reduced with mercaptoethanol and applied on a discontinuous SDS polyacrylamide gel (6-mm slab gel; 7.5% acrylamide). Electrophoresis was run until the tracing dye reached the bottom (14-16 h). The stained gel was photographed before slicing. A plot of the radioactivity distribution measured in slices of the gel strip was aligned with the corresponding Coomassie blue-stained bands. The bromophenol blue dye front corresponds to fraction no. 98.

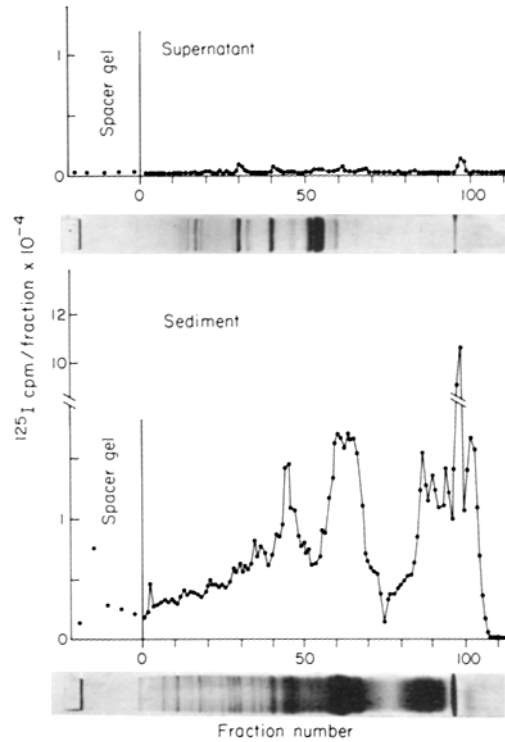


FIGURE 3 Distribution of  $^{125}\text{I}$  radioactivity in microsomal subfractions obtained from RM which were iodinated without addition of detergent. RM incubated with the complete LPO-GO system were recovered and subsequently treated with 0.049% DOC to release the vesicular content. The reduced, processed supernatant ( $40 \mu\text{g}$ ) and sediment ( $400 \mu\text{g}$ ) were loaded onto an SDS discontinuous polyacrylamide gel (6-mm slab gel, 7.5% acrylamide) and analyzed as described in the legend to Fig. 2.

daltons. The peak of 50,000 (between fractions nos. 60 and 70) corresponds to the band identified as cytochrome P-450 by Dehlinger and Schimke (1972). In this gel system most ribosomal proteins migrated near the dye front (fractions nos. 85-100). Only a negligible fraction of the  $^{125}\text{I}$  was incorporated into microsomal lipids since less than 6% of the radioactivity of iodinated microsomes treated on glass filters with hot TCA was removed by subsequent extraction with alcohol/ether (1:1) or chloroform/methanol (3:1).

Iodination in the presence of 0.049% DOC resulted in the labeling of the content fraction as well as the membranes, indicating that when the content proteins were directly exposed to LPO they were readily iodinated (Fig. 4). In this case the radioactivity patterns in gels of supernatants

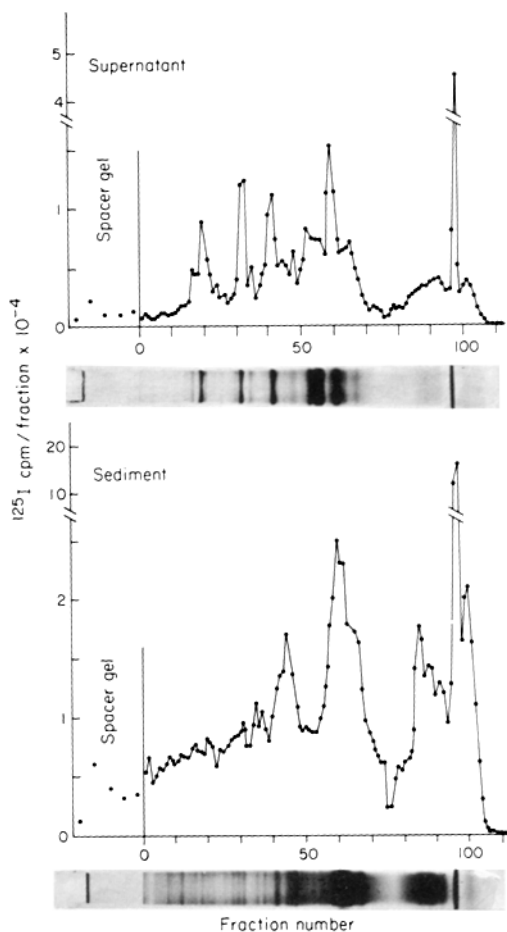


FIGURE 4 Distribution of  $^{125}\text{I}$  radioactivity in microsomal subfractions obtained from RM iodinated in presence of a low concentration of DOC. RM were labeled with  $^{125}\text{I}$  in the presence of 0.049% DOC. After labeling, sediment and supernatant were separated by centrifugation and supernatant proteins were recovered by TCA precipitation. Reduced samples containing 50  $\mu\text{g}$  (supernatant) or 390  $\mu\text{g}$  protein (sediment) were loaded onto 7.5% SDS polyacrylamide gels.

and sediments both coincided with the intensity of the Coomassie blue stain. In addition, the specific activity of the sedimentable membranous subfraction was higher when 0.049% DOC was present (Fig. 4 and Table I).

A comparison of radioactivity patterns in gels from sedimentable membrane fractions showed a uniformly higher level of labeling when iodination was carried out in the presence of 0.049% DOC, although no labeled bands could be resolved which were specifically labeled only in the pres-

ence of detergent. An even higher specific activity of labeling (Table I) was obtained when iodination was carried out in the presence of 0.78% DOC, a detergent concentration which totally dissolved the membrane and solubilized phospholipids (see Fig. 1). However, the radioactivity distribution patterns in gels of total microsomes analyzed after labeling in the presence of 0.049 or 0.78% DOC were qualitatively similar. These observations suggest that most membrane proteins are accessible to iodination from the outer side but are partially buried within the phospholipid matrix. The resolution in one-dimensional gels is not sufficient to exclude labeling of minor bands in the presence of 0.049% DOC which could be either uncovered on the outside by the addition of detergent or be on the inner face of the vesicles and become accessible to the LPO system.

#### Proteolysis of Iodinated Microsomes

It is well known (Kreibich et al., 1973 *a*; Sabatini and Blobel, 1970; Ito and Sato, 1969) that proteolytic enzymes are unable to cross the membrane barrier of intact microsomal vesicles and therefore limit their action to ribosomes and membrane proteins on the outer face. For this reason secretory proteins and polypeptides are protected from digestion by exogenous proteases unless the limiting membrane is made permeable to macromolecules by the addition of low detergent concentrations (0.025–0.049% DOC) (Kreibich et al., 1973 *a*). We found that the addition of detergent during proteolysis had no effect on the extent to which trypsin and chymotrypsin were able to digest proteins of intact microsomes labeled by the LPO system (top part of Fig. 5). Iodinated microsomes which were digested for 3 h at 30°C with or without low detergent concentrations were analyzed on sucrose density gradients. Digestion of intact microsomes led to a decrease in isopycnic density and to a 70% loss of TCA-precipitable radioactivity due to digestion of proteins on the outer face and to release of ribosomes (Fig. 5, panel *b*). Most of the remaining TCA-insoluble radioactivity was associated with the membrane peak (*Mb*) but a small fraction corresponded to iodinated ribosomal proteins of the proteolysed detached ribosomes (radioactivity coincident with the absorbance peak in the upper third of the gradient). A further decrease in isopycnic density resulted when both membrane faces became accessible to the proteases after the

addition of low DOC (Fig. 5, panel *c*). The position of the membrane peak now coincided with that of the released ribosomes (see also Kreibich et al., 1973 *a*). The fraction of  $^{125}\text{I}$ -labeled proteins (~30%) which remained associated with the membranes after proteolysis was unaffected by the addition of detergent (Fig. 5, bottom panels *b* and *c*). This shows that only proteins exposed to

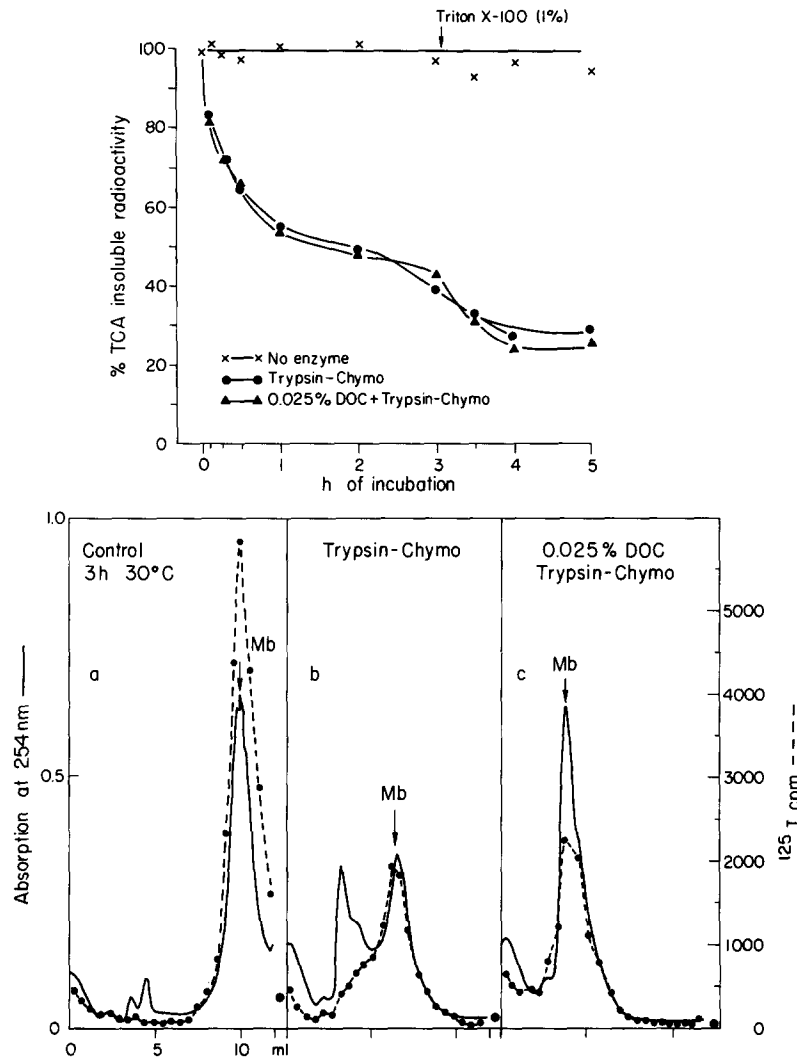


FIGURE 5 Proteolysis of iodinated proteins from the outer face of RM. RM incubated for labeling with  $^{125}\text{I}$  were recovered (30 min, 20,000 *g*, 3°C) and resuspended in HSB (3.5 mg protein/ml). Samples with (▲—▲) or without (x—x, ●—●) 0.025% DOC, received only HSB (x—x) or HSB containing a mixture of trypsin and chymotrypsin (50  $\mu\text{g}/\text{ml}$  of each enzyme) (●—●, ▲—▲), and were incubated at 30°C. At the time points indicated, aliquots (50  $\mu\text{l}$ ) were pipetted onto filter paper disks to measure TCA-precipitable radioactivity. After 5 h (arrow) all samples received Triton X-100 to a final concentration of 0.5% to estimate the maximum digestible material in the sample. Aliquots for zero time values were taken before the addition of enzymes. When necessary, values were corrected for dilution. From all three samples 500- $\mu\text{l}$  aliquots were withdrawn at 3 h which were analyzed in sucrose density gradients (10–50% sucrose containing HSB). After centrifugation (3 h, 200,000 *g*, 3°C). The distribution of TCA-precipitable radioactivity throughout the gradient was measured in 50- $\mu\text{l}$  aliquots processed in filter disks (Kreibich et al., 1973 *a*). The absorbance peak to the left of the labeled membrane in panel *b* represents released and degraded ribosomes.

the outer face of membranes had been previously iodinated.

### Labeling of Microsomes with Pyridoxal Phosphate [ $^3\text{H}$ ]NaBH $_4$

Pyridoxal phosphate was also used in an attempt to label membrane proteins of the outer microsomal face. This reagent reacts with amino groups to form Schiff bases which can be subsequently reduced with tritiated sodium borohydride (Churchich, 1965; Cooper and Reich, 1972). Under the conditions used pyridoxal phosphate is a negatively charged phosphomonoester and should not penetrate the microsomal barrier (Nilsson et al., 1971, 1973). We therefore expected results similar to those obtained with the LPO-catalyzed iodination.

Table II and Fig. 6 show that reduction with [ $^3\text{H}$ ]NaBH $_4$  without pyridoxal phosphate preincubation resulted in low levels of labeling, which in SDS discontinuous acrylamide gels corresponded to a low background and to a fast migrating

TABLE II  
Specific Activity of RM and of Microsomal Subfractions Labeled with Pyridoxal Phosphate and [ $^3\text{H}$ ]NaBH $_4$

	% DOC*	Specific activity $10^7 \times \text{cpm/mg}$ protein	
		37°C†	0°C§
Total RM (omitting pyridoxal phosphate)	0	0.04	0.03
Total RM	0		1.7
Supernatant	0	1.6	1.2
Sediment	0	1.7	1.9
Supernatant	0.025	2.2	2.3
Sediment	0.025	1.9	2.1
Total RM	0.78	2.1	2.4

\* Present during labeling.

† Incubation with pyridoxal phosphate at 37°C; specific activity of [ $^3\text{H}$ ]NaBH $_4$  used, 7.2 Ci/mmol.

§ Incubation with pyridoxal phosphate at 0°C; specific activity of [ $^3\text{H}$ ]NaBH $_4$  used; 5 Ci/mmol. The concentration of pyridoxal phosphate used was 5 times higher than in those at 37°C.

|| RM were recovered after labeling and fractionated with 0.049% DOC (see Material and Methods).

ing compound. A high level of radioactivity was obtained with microsomes pretreated with pyridoxal phosphate at 0°C or at 37°C (Table II). However, tritiated proteins were present in both sedimentable and supernatant subfractions obtained by the low detergent procedure after labeling (Fig. 7). This was in striking contrast to the results obtained when intact RM were enzymatically iodinated. In SDS acrylamide gels the distribution of radioactivity found in both subfractions closely followed the staining pattern. Except for a slightly higher degree of incorporation in the presence of 0.049% DOC the labeling pattern was not affected by the detergent (not shown). As was the case with iodinated microsomes, major tritiated peaks in the sedimentable subfraction coincided with bands corresponding to approximately 50,000, 80,000, and less than 30,000 daltons and with the constituents migrating with the dye front. Since content as well as membrane proteins were labeled in intact microsomes, different digestion kinetics were observed depending on whether a low concentration of DOC (0.025%) was added or not during proteolysis with trypsin and chymotrypsin (Fig. 8, upper panel). After 3 h of incubation approximately 15% more tritium-labeled proteins were digested in the presence of low DOC. Such a difference could be largely accounted for by the labeling of the content (10–15% of the total protein). Sucrose density gradient analysis confirmed these results (bottom panels of Fig. 8).

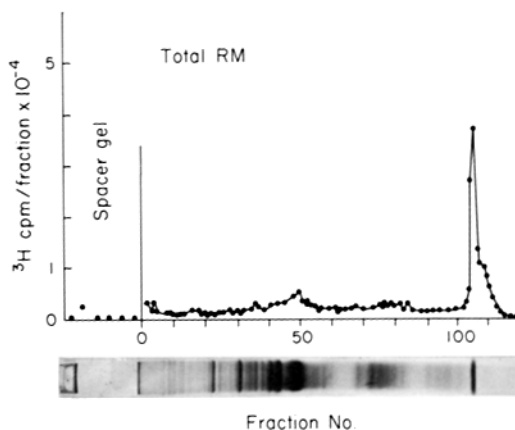


FIGURE 6 Distribution of  $^3\text{H}$  radioactivity in control RM reduced with [ $^3\text{H}$ ]NaBH $_4$ . Total RM were reduced with [ $^3\text{H}$ ]NaBH $_4$  and recovered by sedimentation. A processed aliquot (310  $\mu\text{g}$  protein) was loaded onto a SDS acrylamide gel (6-mm slab gel; 10% acrylamide) which was analyzed as described in the legend to Fig. 2.

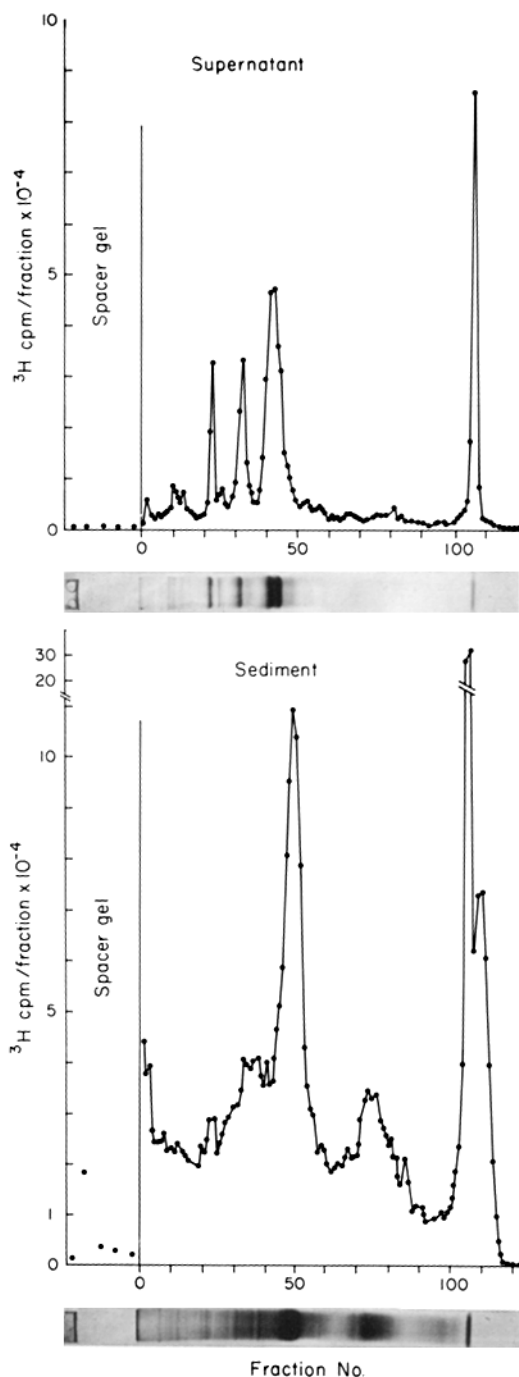


FIGURE 7 Distribution of  $^3\text{H}$  radioactivity in microsomal subfractions from RM incubated pyridoxal phosphate and  $[^3\text{H}]\text{NaBH}_4$ . RM incubated with pyridoxal phosphate (0.2 mM) at  $37^\circ\text{C}$  and subsequently reduced with  $[^3\text{H}]\text{NaBH}_4$  were recovered by sedimentation and treated with 0.049% DOC to release the

## DISCUSSION

The results of this report demonstrate that microsomes are composed of at least two populations of proteins which can be distinguished by a combination of enzymatic iodination and detergent treatment. A class of proteins virtually free of  $^{125}\text{I}$  was released when microsomes previously iodinated as intact vesicles were exposed to a low detergent concentration (0.025% DOC) which altered membrane permeability. The same proteins could be extensively iodinated when microsomes were incubated simultaneously with the LPO system and 0.025% DOC. This demonstrates that microsomal membranes are effective barriers to the iodinating system and reinforces the conclusion (Kreibich et al., 1973 a) that the proteins released by low detergent concentrations represent the cisternal content of the ER which is retained in the vesicular cavities (microsomal content proteins).

We have identified plasma proteins and rapidly labeled glycoproteins within the class of detergent-released material.<sup>1</sup> We also found some released proteins which could only be labeled *in vivo* after a long-term administration of  $[^3\text{H}]\text{leucine}$  and most likely represent long-lived components of the cisternal cavities. In the intact microsomes both types of vesicular proteins were equally inaccessible to the LPO-catalyzed iodination. The detergent concentrations used to release vesicular proteins are insufficient to disassemble the membranes since the membranes can be recovered after sedimentation with a full complement of proteins, phospholipids, and membrane-bound ribosomes. In addition proteolysis of labeled intact microsomes provided further evidence that only proteins exposed on the outer face of the membranes were accessible to LPO-GO iodination. The addition of detergent during proteolysis did not affect the extent to which iodinated proteins were digested.

The labeling of the content in the presence of 0.025% DOC and the concomitantly higher levels of iodination found in the sedimentable membranes suggest that this low level of detergent allowed not only the escape of content but also the entrance of the iodinating system into the open vesicles and labeling of proteins exposed on both sides of the ER membranes. We therefore

vesicular content. Aliquots of supernatant (52  $\mu\text{g}$  protein) and sediment (280  $\mu\text{g}$  protein) were loaded onto 6-mm slab gels of 10% acrylamide which were analyzed as described in the legend to Fig. 2.



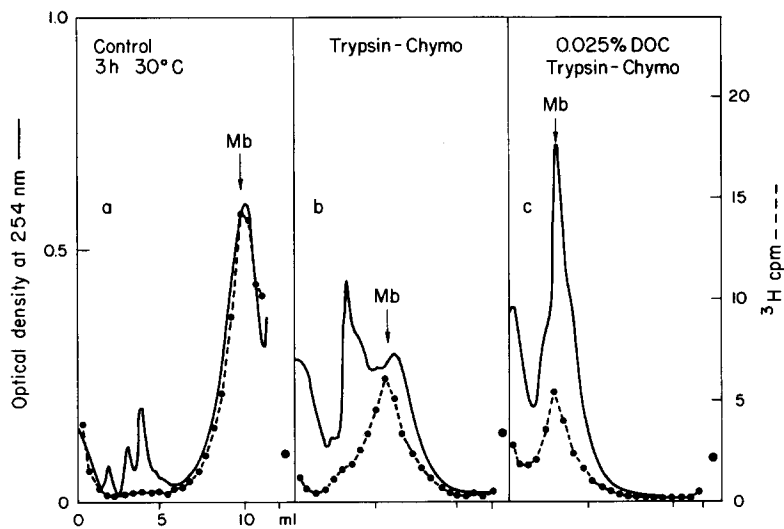
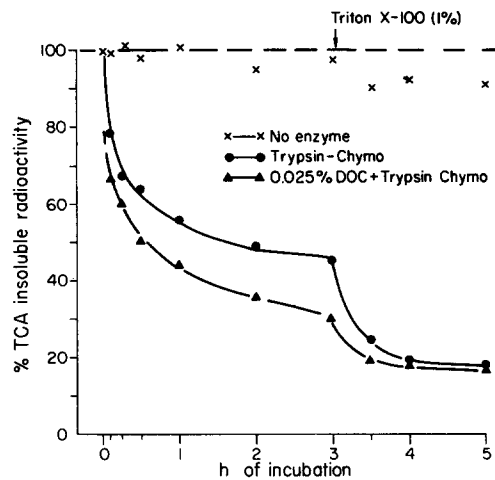


FIGURE 8 Proteolysis of tritiated proteins from RM incubated with pyridoxal phosphate and  $[^3\text{H}]\text{NaBH}_4$ . RM tritiated as intact vesicles (see Fig. 7) were resuspended in HSB (3.5 mg protein/ml) and treated and analyzed as described in the legend to Fig. 5.

attempted to determine the location of proteins by correlating the distribution of  $^{125}\text{I}$  radioactivity to protein patterns in acrylamide gels from microsomes labeled as intact vesicles or labeled as sedimentable vesicles in the presence of 0.025% DOC. However, SDS acrylamide gel analysis revealed that major membrane proteins were already labeled when intact microsomes were incubated with the LPO-GO iodinating system and addition of detergent evidently did not expose previously unavailable major proteins. Thus, we concluded that in intact microsomes most membrane proteins are exposed on that side

of the vesicles which represents the cytoplasmic aspect of ER cisternae. The higher level of labeling of the sedimentable membranes iodinated in the presence of detergent is compatible with the following interpretations: (a) In the ER many membrane proteins are transmembrane proteins, i.e. they span the whole thickness of the ER membrane, and therefore may be labeled from one or both membrane sides. (b) All the additional label acquired by membranes in the presence of detergent is due to uncovering of intramembrane portions of the same proteins which are exposed on the outer face. (c) At least part of the additional

label is incorporated into residual cisternal content which is not extracted when the vesicles are opened by detergent. Although the content can be labeled to a higher specific activity than the membrane proteins, the last possibility alone cannot account for all the increase in labeling, since the remaining content represents no more than 15% of the total protein (Kreibich et al., 1973 a). It cannot be decided from our results, however, whether interpretation (a) or (b) or possibly both apply. Nevertheless in as much as the patterns of labeling do not differ with (low or high) or without detergent treatment it can be concluded that no major membrane proteins containing iodinated tyrosines are exclusively located on the inner membrane face or are completely embedded in the membrane. Gel analysis of higher resolution and double labeling experiments with  $^{125}\text{I}$  and  $^{131}\text{I}$  may serve to elucidate whether inner membrane protein components exist which are not exposed to the cytoplasmic side. Direct evidence of the transmembrane nature of microsomal membrane proteins must in any case come from extensive characterization of a single membrane protein as has been accomplished with the major glycoprotein (glycophorin) in the erythrocyte membrane (Segrest et al., 1973; Bretscher, 1971).

In erythrocyte membranes only glycophorin and a few proteins are accessible to labeling systems applied extracellularly (Phillips and Morrison, 1971; Hubbard and Cohn, 1972; Segrest et al., 1973; Bretscher, 1971). We have found that most microsomal glycoproteins which are detectable in acrylamide gels by periodic acid-Schiff (PAS) staining or after in vivo incorporation of [ $^3\text{H}$ ]glucosamine can be partially released from RM by treatment with low detergent concentrations or by mechanical disruption of the vesicles.<sup>1</sup> By this criterion microsomal glycoproteins behaved as content proteins which are inaccessible to the iodinating system. However these same glycoproteins are less extensively released from open vesicles than other content proteins such as albumin and other plasma proteins<sup>1</sup> (Kreibich et al., 1973 a; Redman and Cherman, 1972). Therefore they could also be regarded as membrane-associated (Fleischer et al., 1971) or peripheral membrane proteins (Singer, 1971) which are weakly associated with the inner face of the membranes. Hirano et al. (1972) have established an inner face location for

microsomal glycoproteins by electron microscopy using ferritin-labeled lectins on membrane fragments from myeloma cell homogenates. Since glycoproteins are quantitatively a minor microsomal component<sup>1</sup> they could not be resolved as individual labeled bands which could be identified as iodinated exclusively from the inner side of vesicles.

Aside from the obvious asymmetry in the location of ribosome binding sites and the asymmetric glycoprotein distribution just mentioned, the extent of proteolytic action on intact or open vesicles has shown that the total amount of protein which is exposed on the outer face of ER membranes is higher than that on the inner face (Kreibich et al., 1973 a). Although it may appear that the asymmetry of microsomal membranes is the inverse of plasma membranes the spatial protein distribution is in fact similar because the inner face of ER membranes can be regarded as topologically equivalent to the outer face of plasma membranes. This follows from the fact that in secretory cells the cisternal content is to be discharged extracellularly (Palade, 1959). This topological equivalence is also compatible with the concept of biogenetic relationships between intracellular membrane systems and the plasma membrane. These may involve processes of circulation, modification, and the possible segregation of membrane components so that basic structural arrangements acquired intracellularly may be fed to the cell surface.

The experiments using the pyridoxal phosphate/[ $^3\text{H}$ ]NaBH<sub>4</sub> labeling procedure indicated that pyridoxal phosphate crossed the microsomal membrane since it labeled the content proteins. In this respect the behavior of pyridoxal phosphate is at variance with that of other charged small molecular weight substrates described by Nilsson et al. (1971, 1973). There were, however, certain restrictions to the passage of pyridoxal phosphate since the final specific activity of labeling in the content fraction was higher when the procedure was carried out in the presence of detergent.

Our results show that the membranes of RM are much more permeable to pyridoxal phosphate than are membranes of influenza virus (Rifkin et al., 1972) derived from the plasma membrane.

We thank Dr. Daniel B. Rifkin for helpful discussions and for information concerning the pyridoxal phosphate-[ $^3\text{H}$ ]NaBH<sub>4</sub> labeling procedure before publica-

tion of the method (Rifkin et al., 1972). The technical assistance of Mrs. Marta Serpa and Miss Belinda Ulrich is gratefully acknowledged.

This work was supported by grants GM 20277-01 and HD 06323-02 from the U. S. National Institutes of Health. Gert Kreibich was a fellow of the Deutsche Forschungsgemeinschaft.

A preliminary report of part of this work was presented at the 57th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., in 1973 (Kreibich et al., 1973 *b*).  
*Received for publication 28 September 1973, and in revised form 7 November 1973.*

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