ENZYME AND PHOSPHOLIPID ASYMMETRY IN LIVER MICROSOMAL MEMBRANES

OLLE S. NILSSON and GUSTAV DALLNER

From the Department of Pathology, Huddinge Hospital, Karolinska Institutet and the Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden

ABSTRACT

The transverse distribution of enzyme proteins and phospholipids within microsomal membranes was studied by analyzing membrane composition after treatment with proteases and phospholipases. Upon trypsin treatment of closed microsomal vesicles, NADH- and NADPH-cytochrome *c* reductases as well as cytochrome b_5 were solubilized or inactivated, while cytochrome P-450 was partially inactivated. When microsomes were exposed to a concentration of deoxycholate which makes them permeable to macromolecules but does not disrupt the membrane, the detergent alone was sufficient to release four enzymes: nucleoside diphosphatase, esterase, β-glucuronidase, and a portion of the DT-diaphorase. Introduction of trypsin into the vesicle lumen inactivated glucose-6-phosphatase completely and cytochrome P-450 partially. The rest of this cytochrome, ATPase, AMPase, UDP-glucuronyltransferase, and the remaining 50% of DT-diaphorase activity were not affected by proteolysis from either side of the membrane. Phospholipase A treatment of intact microsomes in the presence of albumin hydrolyzed all of the phosphatidylethanolamine, phosphatidylserine, and 55% of the phosphatidylcholine. From this observaton, it was concluded that these lipids are localized in the outer half of the bilayer of the microsomal membrane. Phosphatidylinositol, 45% of the phosphatidylcholine, and sphingomyelin are tentatively assigned to the inner half of this bilayer. It appears that the various enzyme proteins and phospholipids of the microsomal membrane display an asymmetric distribution in the transverse plane.

It has been established that several enzymes and glycoproteins of a number of biological membranes such as erythrocytes (56), reticulocytes (38), plasma membranes (17), and inner mitochondrial membranes (29) are not randomly distributed in the transverse plane but are concentrated at the outer or inner surface of these membranes. Bretscher (7) carried out studies with nonpenetrating probes and Zwaal and his coworkers (64) used phospholipases to come to the conclusion that the phospholipids in the erythrocyte membrane are also asymmetrically distributed in the transverse plane.

The membrane of the endoplasmic reticulum (*ER*) contains a large number of enzyme systems, some of them involved in metabolic reactions closely associated with the cytoplasm and some of them participating in the biosynthesis of secretory macromolecules completed and transported within the lumen of the ER and Golgi system (53). Therefore, it is to be expected that, like several other biological membranes, the ER mem-

brane is also asymmetric in its transverse plane. To date, no investigation of the transverse distribution of phospholipids in microsomal membranes has been reported. On the other hand, a number of studies regarding the topology of proteins in this membrane have appeared. The distribution of a number of enzymes was studied by using approaches such as substrate permeability and latency (2, 21, 23, 27, 34), use of charged and uncharged substrates and inhibitors (28), antibodies (1, 34, 35, 46, 48, 51, 59–61), proteolysis (1, 13, 31, 34, 47), and ^{125}I-labeling (63).

Isolated microsomal vesicles are impermeable to various charged substances, which may be the reason why many microsomal enzymes display latency (43). These enzymes apparently possess substrate binding sites at the inner surface of the vesicles or buried in the membrane, and these sites can be made accessible by agents influencing membrane permeability such as detergents or mechanical forces (16). The existence of such a permeability barrier has been established for several microsomal enzymes. Thus, histochemical investigation revealed that inorganic phosphate produced as a result of glucose-6-phosphatase (G6Pase) activity is released in the lumen of the ER (36, 37), while biochemical experiments in vivo and in vitro demonstrated release of the products of several electron-transport enzyme-related reactions on the cytoplasmic side (25, 43).

Very recently, freeze-fracturing of rough microsomes has illustrated an important asymmetry in rough microsomal membranes (45). The electron micrographs showed 80-Å intramembranous particles on the two complementary membrane halves. However, these particles are much more numerous on the cytoplasmic half.

In this paper, experiments are described in which microsomal vesicles were treated with proteolytic and lipolytic enzymes and the effect on microsomal enzymes and phospholipids was studied. By the use of the procedure of Kreibich et al. (33), microsomal vesicles were made permeable to macromolecules, thereby making enzymatic attack on the inside surface also possible. Preliminary accounts of some of these results have appeared elsewhere (42).

MATERIALS AND METHODS

Animals

Adult male albino rats weighing 160–180 g were used. All animals were starved for 20 h before sacrifice. In experiments with ^{32}P, a solution of carrier-free, isotonic [^{32}P]sodium phosphate (Radiochemical Centre, Amersham, England) was injected intraperitoneally (1.2 mCi/100 g body wt) 1 h before decapitation.

Fractionation

Total microsomes and microsomal subfractions were prepared as described previously (12). The microsomal pellet was resuspended (microsomes from 2 g wet weight of liver per 10 ml) in 0.15 M Tris-HCl buffer (pH 8.0), and centrifuged at 105,000 *g* for 60 min to remove adsorbed proteins. The pellets were suspended in 0.25 M sucrose. The protein content of the microsomal suspension was then routinely measured to standardize subsequent incubations.

The permeability of microsomes to macromolecules was increased essentially as described by Kreibich et al. (33) except that magnesium was omitted. Microsomes (40 mg protein in 2 ml 0.25 M sucrose) were added with rapid mixing to 9 ml of medium containing 61 mM KCl, 61 mM Tris-HCl (pH 7.5), 0.25 M sucrose, and 0.061% sodium deoxycholate (DOC). After various incubations, the suspensions were centrifuged without dilution at 105,000 *g* for 2 h, and the pellets were resuspended in 5 ml of 0.25 M sucrose and analyzed.

Enzyme Treatments

The proteolytic enzymes used, trypsin (Boehringer, Mannheim, Germany), α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) and pronase, an unspecific bacterial protease from *Streptomyces griseus* (Sigma Chemical Co.), were dissolved in distilled water (2 mg per ml). They were then dialyzed against 0.05 M Tris-HCl buffer (pH 7.5), at 4°C for 12 h. Where indicated, 50 μg trypsin or pronase or 100 μg α-chymotrypsin per mg microsomal protein was added and incubated at 30°C for 10 min (unless otherwise indicated). All microsomal suspensions, including the controls (without enzymes), were incubated in the same manner. The incubation medium had the same composition as described above to increase the permeability of microsomes to macromolecules, but DOC was omitted when intact microsomal vesicles were analyzed. At the end of the incubation period the samples were cooled in an ice water bath before centrifugation.

Naja-naja snake venom and purified phospholipase A from *Vipera-russelli* (Sigma Chemical Co.) were suspended in 0.05 M Tris-HCl buffer (pH 7.5), at a concentration of 1 mg per ml in the case of *N. naja* and 2 U per ml in the case of the purified phospholipase A. Both phospholipases were then treated at 90°C for 8 min to inactivate proteolytic enzymes, and the protein precipitates were removed by centrifugation. For treatment of microsomes with *N. naja* venom, 0.3 mg of heat-treated venom was added to 40 mg of microsomal protein and in the case of the purified phospholipase A 0.5 U per 40 mg protein was used. The incubation medium also contained

50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, and also routinely 50 mg of bovine serum albumin per ml. Incubations were performed in an ice water bath for 20 min (if not otherwise indicated). The reaction was stopped by the addition of EDTA to give a final concentration of 5 mM.

Phospholipase A_2 from *N. naja* venom (Sigma Chemical Co.) was also purified as described by Cremona and Kearney (10) with the modification of Verkleij et al. (62). The acid- and heat-treated enzyme was gel filtrated on Sephadex G-100. 2 U of the purified enzyme was added to 40 mg of microsomal protein and incubation was performed as described above. Table I shows a comparison of the heat-treated and the purified *N. naja* venom phospholipase on microsomal lipids, using albumin to keep the microsomal vesicles impermeable for macromolecules. The results demonstrate that the effects of the two phospholipase preparations are identical. For this reason, in routine experiments both enzyme preparations could be used.

Phospholipid Analysis

The pellets from the different fractions were extracted with chloroform-methanol, 2:1, in the presence of 0.05% butylated hydroxytoluene (as an antioxidant) and 10 mM EDTA in a nitrogen atmosphere. Partition was performed as described previously (3, 14). Phospholipids (*PL*) were separated by silica gel H thin-layer chromatography according to Parker and Peterson (50). The solvent system consisted of chloroform-methanol-acetic acid-water, 100:60:16:8. The lipid spots were detected with iodine vapor. The appropriate lipid spots were scraped off into scintillation vials, 10 ml of toluol scintillator was added, and radioactivity was measured in a Beckman LS 100 C liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

For chemical measurement of PL, the silica gel samples were extracted with methanol (3 × 30 min at 60°C) and the amount of phosphorus was measured according to Marinetti (40). Since, in our thin-layer procedure, phosphatidylserine and phosphatidylinositol moved as a single spot, these two lipids were extracted after thin-layer chromatography with methanol (3 × 30 min at 60°C), evaporated with a stream of nitrogen, redissolved in chloroform, and fractionated on silica gel-loaded paper (Whatman Ltd., Maidstone, England) as described by Marinetti, using the solvent system diisobutyl ketone-acetic acid-water, 40:25:5, containing 0.05% butylated hydroxytoluene (41). Spots were detected by staining with rhodamine 6 G. The separated lipids were extracted with methanol (3 × 30 min at 60°C) and analyzed for phospholipid and radioactivity.

TABLE I

Comparison of the Effect of Heat-Treated and Purified N. Naja Phospholipase on Intact Microsomal Vesicles

Microsomal phospholipid	Control	Heat-treated *N. naja* venom	Purified *N. naja* venom
		mg PL/g liver	
Sphingomyelin	0.32	0.32	0.31
P-Choline	3.26	1.52	1.49
P-Serine + P-Inositol	1.14	0.69	0.67
P-Ethanolamine	1.54	0.15	0.17

The values shown are means of three experiments.

Permeability

The permeability of microsomes to macromolecules was estimated by using the ultracentrifugation procedure (43). As an indicator of the distribution of macromolecules in relation to the total water and the intramicrosomal water space, [^{14}C]carboxydextran (mol wt 70,000) was used.

Chemical and Enzyme Analysis

Microsomal protein was determined before incubation with enzymes by the biuret reaction (26). In other cases protein was measured according to Lowry et al. (39). Total phospholipid content of the microsomes was analyzed as described previously (14). The various enzyme activities were determined according to previously described procedures (4, 20). The fact that detergent, proteolytic, and lipolytic treatments of microsomes increase several enzyme activities did not influence the results presented in this paper since the procedures for enzymatic analyses were adjusted to obtain maximal activities.

RESULTS

Effect of DOC and Trypsin on Microsomal Permeability

The permeability of microsomal vesicles to dextran of 70,000 mol wt in the presence of increasing concentrations of DOC was checked by using the ultracentrifugation procedure previously applied in studying the permeability properties of microsomes (43). This control was necessary because the preparation procedure for total microsomes was different than that used by Kreibich et al. (33) and also because Mg^{++}, whose presence would have interfered with a number of experiments, was omitted from all media. As an appropriate control for trypsin treatment the microsomes were incubated with DOC at 30°C for 10 min. Under our conditions the microsomes displayed a characteristic permeability change for dextran, essentially in agreement with that seen under the conditions employed by Kreibich et al. (33) (Fig. 1). In

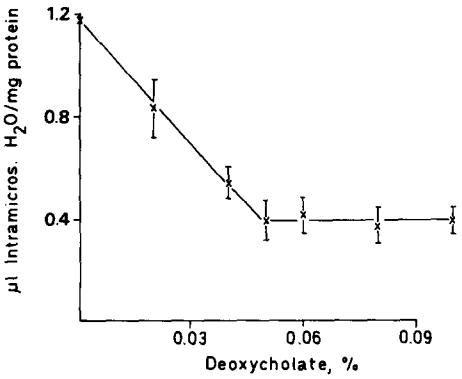

FIGURE 1 Effect of deoxycholate on the permeability of microsomal vesicles to dextran. Tris-washed total microsomes were treated with DOC as described in Materials and Methods. Total and intramicrosomal water and dextran space were determined by using [^{14}C]carboxydextran (mol wt 70,000) as described earlier (43). Nonspecific adsorption was counteracted by adding unlabeled dextran of 70,000 mol wt. The values in the figure represent the size of the intramicrosomal water space not available to dextran. These values are the means of six experiments; the vertical bars represent SEM.

the presence of increasing DOC concentrations, the intramicrosomal water space, to which dextran does not have access, decreases gradually; and taking into consideration water of hydration, it disappears practically completely at a DOC concentration of 0.05%.

Our trypsin treatment of microsomes involved several steps which may damage the microsomal membrane; and, therefore, microsomal permeability to dextran was investigated after various steps. Washing with Tris, which is routinely employed in all experiments, and the water-thermal treatment used to remove secretory proteins affect neither the total water content of the microsomal pellet nor the intramicrosomal water space (Table II, exp. 1). As shown in Table II, exp. 2, trypsin alone does not abolish the microsomal permeability barrier to dextran. The effect of DOC on microsomal permeability is regarded as reversible; and as can be seen in exp. 3, Table II, after removal of DOC by centrifugation the original permeability barrier to dextran is reestablished. Pelleting and resuspension was sufficient to recover the original limited permeability to macromolecules. No washing was needed to remove the remaining DOC for the reestablishment of the original permeability. Increased permeability to dextran could be obtained by adding 0.05% DOC again.

Protein Release by DOC and Trypsin Treatment

The permeability barrier of microsomal vesicles to macromolecules is abolished at a DOC concentration of 0.05%. Therefore, lower detergent concentrations (such as 0.03%) result in only an incomplete release of intravesicular secretory proteins (Table III). It should be noted that treatment in a Mg^{++}-free medium is a significant change of conditions from the original investigation, since in a Mg^{++}-free medium some of the bound ribosomes are detached. In exp. 2, Table III, water-thermal treatment is seen to remove about the same amount of protein as 0.05% DOC. Water-thermal treatment is known to extract luminal secretory proteins, as established previously by measurements of albumin content of the microsomes (24), and, in addition, to release some of the bound microsomes. In the presence of both DOC and trypsin, 55–60% of the total micro-

TABLE II

Effect of Different Treatments on the Permeability of Microsomal Vesicles to Dextran

Experiment	Treatment	Total H_2O	Intramicrosomal H_2O
		µl/mg dry wt	
1	None	2.49	1.18
	Tris wash	2.68	1.35
	Water-thermal	2.62	1.47
2	None	2.54	1.24
	0.05% DOC	2.92	0.43
	Trypsin	2.42	1.52
	0.05% DOC + trypsin	2.90	0.39
3	None	2.41	1.22
	0.05% DOC	2.71	0.41
	0.05% DOC + recentrifugation	2.69	1.26
	0.05% DOC + recentrifugation + 0.05% DOC	2.95	0.25

Microsomes were subjected to the water-thermal treatment as described earlier to remove secretory proteins (12). Treatments with DOC and trypsin are described in Materials and Methods. The intramicrosomal water represents the water space which is not accessible to dextran (70,000 mol wt). Estimation of the water spaces was performed by the ultracentrifugation procedure (43). In experiment 3, the DOC-treated microsomes in 11 ml were recentrifuged at 105,000 *g* for 2 h without dilution. The pellet was resuspended in 11 ml of 0.25 M sucrose, and the intramicrosomal water space was estimated. The values are the means of three experiments.

TABLE III

Effect of Proteases on Microsomal Membranes

Experiment	Treatment	Protein	PL
		mg/g liver	
1	None	16.0	6.3
	0.03% DOC	14.2	6.3
	0.05% DOC	11.2	6.1
	Trypsin	10.2	6.4
	Chymotrypsin	10.0	5.9
	Pronase	10.2	6.1
	Trypsin + 0.05% DOC	7.0	5.9
	Chymotrypsin + 0.05% DOC	7.5	5.8
	Pronase + 0.05% DOC	7.2	6.1
2	None	16.0	6.5
	Water-thermal	11.7	6.3

In exp. 1, microsomes were incubated in the presence of DOC and/or various proteases. At the end of the incubation (10 min, 30°C) the fractions were centrifuged (105,000 *g*, 120 min) and the pellets were suspended in 0.25 M sucrose for protein and phospholipid analysis. In exp. 2, water-thermal treatment was performed for comparison. The values are the means of three experiments.

somal protein is solubilized. Since both DOC and trypsin release, at least in part, the same microsomal proteins (such as ribosomes and a few microsomal enzymes as discussed later), it is difficult to judge on the basis of these experiments how much of the inner surface protein is hydrolyzed by trypsin. However, on the basis of protein, phospholipid, and RNA measurements, we calculate that 6–11% of the microsomal protein solubilized by trypsin in the presence of DOC originates from the inner surface. Two more proteolytic enzymes, chymotrypsin and unspecific protease, were also tested, and the results, both in the absence and in the presence of DOC, were the same as with trypsin. In all of the above experiments phospholipids were also determined and there was no evidence of lipid release in any of the treatments employed.

Effect of Proteolysis on Enzymes

The transverse distribution of various electron-transport enzymes in microsomal membranes was investigated by using the DOC-trypsin approach. It appears that not all of these enzymes are distributed in the same manner (Table IV). Treatment with 0.05% DOC influences only one enzyme to a significant extent, i.e., about 40% of the DT-diaphorase activity is released into the supernate. Treatment with trypsin, chymotrypsin, or protease solubilizes NADPH-cytochrome *c* reductase activity completely, but DT-diaphorase activity is practically uninfluenced by proteolysis. The cytochrome P-450 amount clearly decreases after treatment with proteolytic enzymes. The appearance of a peak at 420 nm in the difference spectrum indicates an inactivation of the cytochrome, but recovery of cytochrome P-450, even taking into consideration this peak, is not complete. This cytochrome is further inactivated when the proteases are allowed access to the luminal compartment with DOC. DT-diaphorase is not significantly influenced by the combined DOC + trypsin treatment. The solubilized part of both NADPH-cytochrome *c* reductase and DT-diaphorase was recovered from the supernate in active form.

The behavior of the NADH-oxidizing enzymes is shown in Table V. The marked inactivation of NADH-cytochrome *c* reductase activity by proteolytic treatments may be due to the solubilization of cytochrome b_5. NADH-ferricyanide reductase activity is uninfluenced by proteolysis, whereas

TABLE IV

Distribution of NADPH-Linked Enzymes and DT-Diaphorase in Microsomal Membranes

Treatment	NADPH-Cytochrome *c* reductase	Cytochrome P-450	DT-Diaphorase
	% control		
None	100 (0.11)[1]	100 (1.67)[2]	100 (0.06)[1]
0.03% DOC	100 ± 13	100 ± 18	95 ± 6
0.05% DOC	98 ± 13	87 ± 9	62 ± 5
Trypsin	12 ± 1.9	59 ± 4	89 ± 9
Trypsin + 0.05% DOC	12 ± 1.7	29 ± 3	49 ± 5
Chymotrypsin	15 ± 1.6	54 ± 4	91 ± 7
Chymotrypsin + 0.05% DOC	15 ± 2.1	25 ± 4	52 ± 4
Pronase	11 ± 1.0	53 ± 7	90 ± 12
Pronase + 0.05% DOC	11 ± 1.3	26 ± 3	56 ± 8
Recovery			
0.05% DOC	99 ± 12	88 ± 7	90 ± 12
Trypsin	91 ± 10	66 ± 4	88 ± 9
Trypsin + 0.05% DOC	89 ± 14	34 ± 7	87 ± 6

The values in brackets give the specific activities in micromoles NADPH oxidized per minute per milligram PL[1] or nanomoles cytochrome P-450 per milligram PL[2]. The absolute activities and amounts found in control microsomes, which were incubated in 0.25 M sucrose at 30°C for 10 min and also recentrifuged, were taken as 100%. The remaining activity or amount in the recentrifuged pellet after the various treatments was related to the control. The supernate, after centrifugation, was also analyzed, and the sum of the activity in the pellet and supernate was used to calculate the recovery. The values are means ± SEM (n = 7).

more than two-thirds of the microsomal cytochrome b_5 is solubilized. The remaining cytochrome b_5 is practically eliminated by the combined DOC + trypsin incubation with a concomitant decrease in NADH-cytochrome *c* reductase activity. NADH-ferricyanide reductase is only slightly influenced by the combined treatment. Difference spectroscopy demonstrated a full recovery of the solubilized cytochrome b_5 from the supernate.

None of the microsomal phosphatase activities are significantly influenced by the proteases employed, with the possible exception of adenosine triphosphatase (ATPase) activity, which is slightly inactivated (Table VI). DOC treatment solubilizes inosine diphosphatase (IDPase) activity completely. A similar clear-cut effect of proteases in the presence of DOC on G6Pase activity may be observed. This enzyme, which is completely unaffected by protease treatment of intact microsomes, is fully inactivated when the proteases act on permeable vesicles. Adenosine monophosphatase (AMPase) activity is apparently buried in the membrane in such a way that it is protected from proteolysis, both in the presence and in the absence of DOC. ATPase, which is slightly affected by proteolysis of the intact vesicles, exhibits a further small decrease of activity when the proteases are introduced into the vesicle lumen. Probably, this limited decrease in activity is caused by inactivation of the enzyme, since it is known to tolerate very poorly any treatment with detergents and centrifugation (23).

Microsomal esterase activities measured with *p*-nitrophenylacetate and *p*-nitrophenylproprionate as substrates as well as β-glucuronidase activity are almost completely solubilized from microsomes by increasing the permeability to macromolecules with 0.05% DOC,[1] in contrast to uridine diphospho-glucuronic acid (UDPGA)-transferase activity which is not solubilized to a significant extent (Table VII). None of these enzymes are decreased in activity by protease treatment in the absence of DOC. Like ATPase and AMPase, UDPGA-transferase activity is affected only to a limited degree when the proteolytic enzymes are introduced into the luminal compartment. Esterase and β-glucuronidase activities released from the microsomes were recovered from the supernate in active form in all cases.

[1] Secretory proteins were also removed from microsomes with the water-thermal treatment, which did not cause a substantial loss of esterase activity. Therefore, this enzyme activity measured under our conditions is not associated with albumin.

TABLE V

Distribution of NADH-Linked Enzymes in Microsomal Membranes

Treatment	NADH-Cytochrome *c* reductase	NADH-ferricyanide reductase	Cytochrome b_5
	% control		
None	100 (1.75)[1]	100 (3.53)[1]	100 (1.98)[2]
0.03% DOC	82 ± 5	80 ± 11	100 ± 9
0.05% DOC	80 ± 6	75 ± 8	100 ± 12
Trypsin	40 ± 5	85 ± 9	30 ± 4
Trypsin + 0.05% DOC	18 ± 3	62 ± 5	5 ± 1.5
Chymotrypsin	45 ± 4	92 ± 10	28 ± 2.2
Chymotrypsin + 0.05% DOC	25 ± 3	68 ± 4	8 ± 1.5
Pronase	30 ± 4	89 ± 9	21 ± 3
Pronase + 0.05% DOC	15 ± 2.4	70 ± 8	5 + 1.1
Recovery			
0.05% DOC	81 ± 10	75 ± 6	100 ± 12
Trypsin	43 ± 4	87 ± 9	91 ± 11
Trypsin + 0.05% DOC	19 ± 3	65 ± 10	90 ± 9

The values in brackets give the specific activities in micromoles NADH oxidized per minute per milligram PL[1] or nanomoles cytochrome b_5 per milligram PL[2]. Otherwise as in Table IV. The values are means ± SEM (n = 7).

TABLE VI

Distribution of Phosphatase Activities in Microsomal Membranes

Treatment	ATPase	AMPase	IDPase	G6Pase
	% control			
None	100 (0.36)[1]	100 (0.20)[1]	100 (1.89)[1]	100 (1.05)[1]
0.03% DOC	98 ± 13	96 ± 14	65 ± 6	111 ± 13
0.05% DOC	97 ± 12	98 ± 15	11 ± 1.9	72 ± 7
Trypsin	81 ± 8	95 ± 11	95 ± 12	98 ± 8
Trypsin + 0.05% DOC	67 ± 6	92 ± 10	8 ± 1.7	12 ± 2.2
Chymotrypsin	81 ± 10	95 ± 9	94 ± 12	97 ± 9
Chymotrypsin + 0.05% DOC	67 ± 7	92 ± 9	9 ± 1.4	14 ± 1.9
Pronase	78 ± 7	93 ± 12	93 ± 8	95 ± 10
Pronase + 0.05% DOC	71 ± 9	95 ± 13	8 ± 1.6	15 ± 1.9
Recovery				
0.05% DOC	97 ± 13	98 ± 9	95 ± 13	82 ± 4
Trypsin	84 ± 11	94 ± 11	96 ± 9	98 ± 7
Trypsin + 0.05% DOC	69 ± 8	94 ± 13	94 ± 10	26 ± 3

The values in brackets give the specific activities in micromoles P_i per minute per milligram PL[1]. Otherwise as in Table IV. The values are means ± SEM (n = 8).

Time of Proteolysis

Several considerations must be taken into account when the effect of hydrolytic enzymes on

membrane structure is being studied. One is that the enzyme in question does not have a very limited substrate specificity. Microsomal membranes contain a large number of enzymes and other proteins which may create a problem when proteolytic enzymes with rather specific substrate specificity are used. However, the three proteases used here, among them the unspecific bacterial protease, did not give significantly different results.

Another consideration is that the hydrolytic enzymes must be incubated with the membranes long enough to give maximal effect. Some of the microsomal surface proteins may be hydrolyzed relatively slowly. This possibility was controlled by using prolonged incubation time with the three enzymes. Since chymotrypsin and protease gave results practically identical to those obtained with trypsin, only the latter are shown in Fig. 2. After a 10-min incubation of total microsomes with trypsin, about 35% of the microsomal protein is solubilized and this value did not increase upon further incubation (Fig. 2a). G6Pase, esterase, and UDPGA-transferase (which are affected only slightly, if at all, by trypsin treatment) display similar activities after 20 and 30 min of incubation, as after 10 min. In addition, NADPH-cytochrome *c* reductase is maximally affected after 10 min of hydrolysis. In the presence of 0.05% DOC the situation was very similar, that is, in no case was enzyme release or inactivation enhanced by prolonged incubation (Fig. 2b). All of the enzymes shown in the various tables were analyzed in this fashion, and in all cases full effect was observed after a 10-min incubation with protease.

TABLE VII

Distribution of Esterase, β-Glucuronidase, and UDPGA-Transferase Activities in Microsomal Membranes

Treatment	*p*-NP-Acetate esterase	*p*-NP Propionate esterase	β-Glucuronidase	UDPGA-Transferase
		% control		
None	100 (12.6)[1]	100 (23.8)[1]	100 (0.19)[1]	100 (0.018)[2]
0.03% DOC	78 ± 7	85 ± 11	94 ± 10	95 ± 11
0.05% DOC	26 ± 3	38 ± 4	28 ± 4	82 ± 7
Trypsin	98 ± 9	96 ± 10	82 ± 8	90 ± 7
Trypsin + 0.05% DOC	24 ± 4	34 ± 5	15 ± 2.8	74 ± 6
Chymotrypsin	97 ± 11	98 ± 10	72 ± 6	94 ± 11
Chymotrypsin + 0.05% DOC	22 ± 3	26 ± 3	18 ± 1.5	68 ± 9
Pronase	99 ± 8	102 ± 14	70 ± 6	91 ± 10
Pronase + 0.05% DOC	27 ± 4	33 ± 4	14 ± 1.5	69 ± 8
Recovery				
0.05% DOC	89 ± 8	91 ± 8	96 ± 10	80 ± 7
Trypsin	97 ± 10	98 ± 9	84 ± 8	91 ± 7
Trypsin + 0.05% DOC	90 ± 11	87 ± 6	83 ± 9	78 ± 8

The values in brackets give the specific activities in micromoles *p*-nitrophenyl liberated per minute per milligram PL[1] or micromoles *p*-nitrophenyl conjugated per minute per milligram PL[2]. Otherwise as in Table IV. The values are means ± SEM (n = 7). *NP* = nitrophenyl.

Microsomal Subfractions

One possible explanation for the effect of proteases on several microsomal enzymes is that these

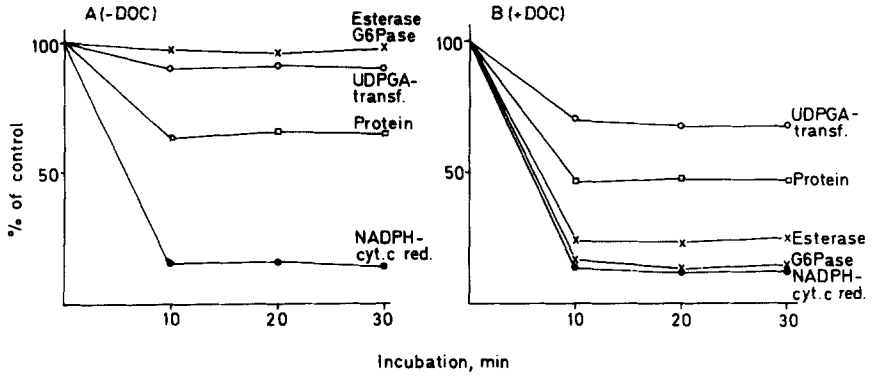

FIGURE 2 Time-course of proteolysis of microsomal enzymes. Microsomes were incubated with trypsin (50 μg per mg microsomal protein). Incubations were performed, as described in Materials and Methods, in the absence (A) or in the presence (B) of 0.05% DOC. After various time periods, the suspension was cooled and centrifuged (105,000 *g*, 120 min). After resuspension of the pellets, protein and enzyme activities were determined. The absolute activities found in control microsomes, which were incubated in the same incubation medium at 30°C for 10 min and also recentrifuged, were taken as 100%. The values are the means of five experiments.

activities in certain microsomal membranes but not in others are affected by this treatment. In this case the observed result would be a mixture of the results for the two populations. To test this point, rough and smooth microsomes were separated and treated separately with DOC and trypsin (Table VIII). There were some variations in enzyme activities released or inactivated by proteolysis of the two subfractions, but these variations were far from sufficient to change our conclusions concerning the asymmetric distribution of the various enzymes in the transverse plane of the ER membrane. The recovery values from the supernate were essentially the same as described for total microsomes.

Attempt to Prepare Inside-Out Vesicles

The reliability of the experiments described above could be increased by performing proteolysis on inside-out vesicles, where the luminal surface is attacked directly without using detergents. Since sonication produces extensive breakup of microsomal vesicles into smaller vesicles (11), and since sonication is used to prepare inside-out inner mitochondrial membranes (29), microsomes were subjected to sonication. As in the nonsonicated microsomes, trypsin treatment of these submicrosomal particles removed all of the NADPH-cytochrome *c* reductase and none of the G6Pase activity (Table IX). Obviously, the sonication created small vesicles, all of them with the same outside-out orientation as in nonsonicated microsomes.

Effect of Phospholipase Treatment on Microsomal Enzymes

To obtain further insight into the distribution of microsomal enzymes, various activities were measured after treatment of microsomes with two phospholipases and with *V. russeli* phospholipase and trypsin together. These treatments were performed either in the presence or in the absence of albumin since, as will be shown later, the presence of albumin prevents penetration of the microsomal membrane by phospholipases.

Phospholipase treatment hydrolyzed a large part of the microsomal PL; and the products, lysophospholipids and fatty acids, were removed by centrifugation in the presence of albumin (Table X). Part of the lysophospholipids remained in the microsomal pellet when incubation was performed with venom from *N. naja* in the absence of albumin. G6Pase, IDPase, DT-diaphorase, and esterase activities were only slightly influenced when phospholipase treatment, in the presence or absence of trypsin, was performed in the presence of albumin. On the other hand, G6Pase was inactivated, and IDPase, DT-diaphorase, and esterase activities were solubilized in active form when venom from *N. naja* was used for the treatment in the absence of albumin. This is probably a result of the detergent effect of the nonbound lysophospholipids. In this case, the phospholipase gains

TABLE VIII

Effect of Proteolysis and DOC on Enzyme Activities in Rough and Smooth Microsomes

Enzyme analyzed	0.05% DOC	Trypsin	Trypsin + 0.05% DOC
		% control	
Rough microsomes			
Protein	71	65	45
p-NP-Acetate esterase	19	86	33
p-NP-Propionate esterase	35	86	29
β-Glucuronidase	35	81	18
NADH-cytochrome *c* reductase	79	32	11
NADPH-cytochrome *c* reductase	100	11	13
NADH-ferricyanide reductase	83	83	70
IDPase	11	73	5
G6Pase	95	87	23
DT-Diaphorase	39	102	41
Smooth microsomes			
Protein	76	80	52
p-NP-Acetate esterase	33	90	33
p-NP-Proprionate esterase	36	118	32
β-Glucuronidase	30	76	21
NADH-cytochrome *c* reductase	75	35	11
NADPH-cytochrome *c* reductase	100	8	13
NADH-ferricyanide reductase	77	82	79
IDPase	14	79	10
G6Pase	84	100	34
DT-Diaphorase	54	98	40

Rough and smooth microsomes were Tris-washed and treated with DOC and trypsin in the same manner as total microsomes. The values in the control microsomes were taken as 100%. The values given represent the means of three experiments. *NP* = nitrophenyl.

access to the inner compartment of the vesicle (as will be shown later in Table XI).

Enzyme inactivation may occur either as a result of removal of phospholipids or as a result of inhibition by residual lysophospholipids or fatty acids. UDPGA-transferase is largely inactivated in the presence of trypsin and phospholipase or when treatment with venom from *N. naja* is carried out in the absence of albumin. 60% of the β-glucuronidase activity is abolished from the pellet by the treatment with venom from *N. naja* in the absence of albumin, but it was recovered in the supernate. The inactivation of NADH-cytochrome *c* reductase with *V. russeli* phospholipase, particularly in the presence of trypsin, may be explained by the very effective solubilization of cytochrome b_5 under these conditions. The results with phospholipases thus further support the enzyme distribution pattern established with the use of trypsin and a low concentration of DOC.

TABLE IX

Sonication of Microsomes

Fraction	Treatment	NADPH-Cytochrome *c* reductase	G6Pase
		μmol NADPH ox/min/mg PL	*μmol P_i/min/mg PL*
Microsomes	None	0.12	1.01
	Trypsin	0.012	1.00
Sonicated microsomes	None	0.10	0.93
	Trypsin	0.009	0.90

The microsomes were sonicated with a Branson sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) (80 mg protein in 4 ml 0.25 M sucrose for 5 min at 2 Å), and then trypsin treatment was performed as described in Materials and Methods. The nontreated and trypsin-treated microsomes and sonicated microsomes were sedimented by centrifugation (105,000 *g*, 4 h). Enzyme activities were measured in the suspended pellet. The values are the means of three experiments.

Effect of Phospholipase Treatment on Permeability

Phospholipase A from *V. russeli* liberates free fatty acids and diglycerides from PL, whereas phospholipase A_2 from *N. naja* venom liberates both fatty acids and lysophospholipids. In the presence of relatively large amounts of albumin, these products are bound, cannot exert their detergent-like effect, and can be separated from microsomal membranes by centrifugation. This fact is demonstrated in Table XI. In the absence of albumin, both phospholipases not only hydrolyze a large part of the PL but also make the intramicrosomal water space accessible to high molecular weight dextran.

Enzymic Hydrolysis of Phospholipids

To study the intramembranous distribution of phospholipids, liver microsomes prepared from rats injected with ^{32}P were incubated with *N. naja*

TABLE X

Effect of Phospholipase Treatment on Enzyme Activities of Microsomal Membranes

Component	*V. russeli* pellet	*N. naja* pellet	*V. russeli* + trypsin pellet	*N. naja* − BSA	
				Pellet	Supernate
			% control		
NADPH-cytochrome *c* reductase	100	83	17	53	38
NADH-cytochrome *c* reductase	23	45	8	64	9
NADH-ferricyanide reductase	93	100	63	80	5
Acetate esterase	55	70	58	17	80
β-Glucuronidase	61	80	72	44	48
UDPGA-transferase	57	65	33	20	31
G6Pase	65	64	57	4	11
IDPase	83	95	62	2	89
DT-Diaphorase	107	64	64	23	92
Phospholipid	36	44	31	56	44

Phospholipase treatments were performed as described in Materials and Methods except for the *N. naja*-BSA experiment, where bovine serum albumin (*BSA*) was omitted from the medium. When trypsin was added to the *V. russeli* phospholipase A-containing medium, the amount of trypsin was 50 μg/mg microsomal protein. The values are the means of four experiments.

TABLE XI

Effect of Phospholipase Treatment on Microsomal Permeability

Treatment	Albumin	PL	Total H_2O	Intramicrosomal H_2O
	mg/ml in medium	*mg/g liver*	*μl/mg dry wt*	
None	0	6.3 ± 0.4	2.7 ± 0.3	1.19 ± 0.09
V. russeli venom	0	3.2 ± 0.2	3.0 ± 0.3	0.54 ± 0.07
V. russeli venom	50	2.5 ± 0.2	1.9 ± 0.2	1.03 ± 0.10
N. naja venom	0	3.6 ± 0.3	3.1 ± 0.2	0.49 ± 0.08
N. naja venom	50	2.9 ± 0.2	2.0 ± 0.2	1.13 ± 0.11

Incubations were performed in the presence or absence of 50 mg bovine serum albumin per ml incubation medium. Phospholipid determination as earlier (14). Measurement of water spaces as in Table II. The values are means ± SEM (n = 6).

phospholipase A_2 in the presence of albumin. After thin-layer chromatography the radioactivity in the individual spots was determined (Fig. 3). As expected, sphingomyelin was not hydrolyzed by phospholipase A_2. 45% of the phosphatidyl(P)-serine and P-inositol, which appears as a single spot in the system used here, was recovered unhydrolyzed in the microsomes. After separation of these two phospholipids on paper impregnated with silica gel, however, two very different behaviors became apparent: 91% of P-inositol but only 12% of the original P-serine was recovered in the microsomal pellet. P-Choline, the largest phospholipid component of microsomal membranes, was partly recovered (47%); and P-ethanolamine, like P-serine, was almost completely (90%) hydrolyzed. According to these results, 45% of the total PL remained in the membrane and 55% was hydrolyzed and released. After 20 min of incubation no further hydrolysis of phospholipids was seen.[2]

Similar results for phospholipid distribution were obtained when microsomes were incubated with phospholipase A from *V. russeli*. However, as seen previously with erythrocytes, it was more difficult to control the activity of this enzyme, even in the presence of albumin.[3]

The results observed with phospholipase A from *N. naja* venom cannot be explained by the inability of this enzyme to hydrolyze certain phospholipids. If albumin was omitted from the incubation medium, the lysocompounds could exert their detergent effect, and the phospholipase completely hydrolyzed all of the phospholipids except sphingomyelin.

The distribution of sphingomyelin was studied by using sphingomyelinase C (Table XII). The effectivity of enzyme is demonstrated by the fact that sphingomyelin micelles of pig brain and of rat liver microsomes were almost completely hydrolyzed after a 15-min incubation. Intact microsomes in the presence of albumin were not attacked by the enzyme, while in the case of permeable microsomes (0.05% DOC in the medium), 80% of the sphingomyelin was hydrolyzed. Thus, these experiments suggest that this lipid is distributed at the inner half of the lipid bilayer.

For routine analysis, microsomes from rats treated with $^{32}P_i$ were used and the radioactivity in the individual spots was determined after thin-layer chromatography. In separate experiments, however, the results were confirmed by measuring the phosphorus content of the individual spots. Table XIII demonstrates that these two methods give the same results. After removal of those phospholipids regarded as situated in the outer half of the bilayer, the specific radioactivity of the remaining phospholipids is essentially the same as the original sp act. This experiment shows that the lipid compartment removed does not represent a specific fraction into which the precursor used is especially rapidly incorporated.

DISCUSSION

The experiments performed in this investigation suggest that, in addition to the well-documented

[2] Treatment with *N. naja* venom was also carried out with isolated rough microsomes instead of the total microsomal fraction. There was no difference in the two distribution patterns.

[3] Phospholipase treatments were also repeated with 50 μg trypsin/mg protein in the incubation medium. In these experiments the results were the same as without trypsin, that is, no additional hydrolysis of the individual phospholipids was obtained.

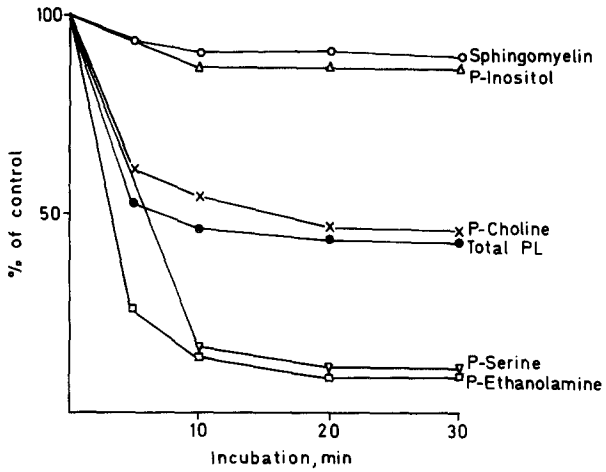

FIGURE 3 Effect of treatment with *N. naja* venom on microsomal phospholipids. Microsomes were prepared from rats injected 60 min before decapitation with carrier-free ^{32}P intraperitoneally (1.2 mCi/100 g body wt). The phospholipase treatment, extraction, and analysis of phospholipids were performed as described in Materials and Methods. The values are given as percent of radioactivity in the pellet after treatment relative to the control. The values are the means of six experiments. P = phosphatidyl.

lateral asymmetry of the endoplasmic reticulum (16), a transverse asymmetry of many protein and phospholipid components also exists. The distribution of the various components in the membrane can, however, only be given as a tentative distribution at present, since the methods employed to study asymmetry possess inherent limitations. These investigations utilize hydrolytic enzymes for selective breakdown of the membrane, and the inside surface of the vesicle is made available for the enzymic attack by a detergent procedure. This is perhaps not an ideal approach.

It is obvious that the distinction between outer, inner, and central compartments is operational. The proteases used have a certain specificity, and certain peptide bonds on the surface may be located unfavorably for hydrolysis, e.g., because of protection by neighboring components as has been demonstrated for the β-segment of glycophorin in the erythrocyte membrane (32). In addition, the active site represents only a small portion of an enzyme, and other portions of the molecule may extend relatively far from this site. Trypsin treatment removes the heme-containing catalytically active part of the cytochrome b_5, while the noncatalytic hydrophobic unit, possessing only a structural role, is not affected by proteolytic treatment (55). Also, release of a peptide sequence from an enzyme protein does not necessarily affect the catalytic center, which may not be available for enzymic hydrolysis. Most of the enzyme treatments require incubation at elevated temperature, which may activate endogenous microsomal hydrolytic enzymes. Even in the absence of added divalent cations, isolated microsomes contain sufficient amounts of bound calcium to activate, for example, endogenous phospholipase A_1, an enzyme that can hydrolyze the membrane in which it is located (6). Careful control experiments are therefore basic to such studies, particularly when prolonged incubations are required. Finally, it is possible that removal of protein components by proteolysis leads to reorganization of the membrane so that, for example, previously buried components become exposed at the outer or inner surface of the membrane.

The low detergent-salt procedure of Kreibich et al. (33), which makes the microsomal membrane permeable to macromolecules without disrupting its structure, is a very useful technique for studying the transverse structure of microsomal vesicles. Various types of enzymes can be introduced into the vesicular compartment and thereby digest components localized on the inner surface. This procedure makes it possible to perform a large number of different studies. However, these studies do, of course, involve a number of possible artefacts. It may well happen that DOC brings about effects other than that of increasing permeability, such as breaking up protein-protein or protein-lipid interactions. Such an effect could thus explain increased proteolysis of a membrane com-

TABLE XII

Sphingomyelinase Treatment of Microsomes

Fraction	Treatment	Sphingomyelin in fraction	
		mg	% non-treated
Sphingomyelin (pig brain)	None	3.0	
	Sphingomyelinase	0.45	15
Sphingomyelin (microsomal)	None	1.50	
	Sphingomyelinase	0.29	19
Microsomes	None	0.90	
	Sphingomyelinase	0.83	92
Permeable microsomes	None	0.90	
	Sphingomyelinase	0.19	21

Pig brain sphingomyelin (Serdary Research Lab., Inc., London, Ontario, Canada) and liver microsomal sphingomyelin, isolated by preparative thin-layer chromatography, were sonicated (10 mg in 5 ml 50 mM Tris-HCl, pH 7.5, 10 mM KCl for 5 min at 2 Å) with a Branson sonifier to obtain micelles. After centrifugation (105,000 *g*, 20 min), the supernate was used. Microsomes and permeable microsomes (0.05% DOC-treated) were prepared as described in Materials and Methods. The fractions were incubated with 4 IU sphingomyelinase C (*S. aureus*), kindly provided by Dr. R. F. A. Zwaal (State University of Utrecht, The Netherlands). The incubation solution, besides enzyme and lipid fractions, contained 50 mM Tris-HCl, pH 7.5; 5 mM $CaCl_2$; 0.25 M $MgCl_2$; and 10 mM KCl in a final volume of 10 ml. In the case of microsomes and permeable microsomes, 0.5 g albumin was also included. Incubation was performed at 30°C for 15 min. The values are based on three experiments.

ponent. The detergent concentration used in these experiments is far below what is necessary for solubilization, but it may still remove components masking an underlying enzyme or cause membrane reorganization which in exceptional cases could expose previously inaccessible sites.

The most reliable investigation so far on membrane asymmetry was performed on erythrocytes (56, 64) and inner mitochondrial membranes (29), since in these cases it is possible to produce inside-out vesicles. It is claimed that such inside-out transformation occurs in 15% of the vesicles isolated from the sarcoplasmic reticulum of muscle (8). It is improbable that we have this problem in the case of liver microsomes. The morphological appearance, together with the fact that some enzymes such as NADPH-cytochrome *c* reductase are completely removed by proteolytic treatment whereas others such as IDPase are completely retained, strongly indicates that all microsomal vesicles are of outside-out type. We made an attempt to prepare inside-out vesicles by intensive sonication, which creates small vesicles in the range 300–1,100 Å (11). However, it appears that even this extensive breakage is insufficient to change the transverse orientation of the vesicles.

Expressed in a highly simplified and schematic manner, NADPH-cytochrome *c* reductase, cytochrome b_5, and probably at least part of the GDP-mannose-transferase system (13) are located on the cytoplasmic surface of the microsomal membranes. Obviously, different proteins are bound to the inner side of the membrane in different manners; both loosely and tightly bound enzymes are present. At the inner surface, we find nucleoside diphosphatase, esterase, microsomal β-glucuronidase, and glucose-6-phosphatase. AMPase and UDPGA-transferase appear to be buried in the membrane, well-protected from trypsin attack from both sides, unlike other microsomal enzymes. ATPase is inactivated to a small extent by trypsin treatment of intact vesicles but it is uncertain to what extent the localization of this enzyme differs from that of AMPase, for instance. Cytochrome P-450, the enzyme present in the microsomal membrane in the largest amount, is unique

TABLE XIII

Effect of Lipolysis on Specific Radioactivity of Microsomal Phospholipids Isolated from In Vivo Labeled Rat Liver

Phospholipid	Control		*N. naja* venom	
	mg PL/g liver	*cpm/mg PL*	*mg PL/g liver*	*cpm/mg PL*
Sphingomyelin	0.34	17,000	0.32	16,900
P-Choline	3.26	200,600	1.60	194,000
P-Serine + P-inositol	1.13	65,300	0.60	60,500
P-ethanolamine	1.51	355,300	0.19	318,800

Rats injected intraperitoneally with carrier-free ^{32}P (1.2 mCi/100 g body wt) were decapitated 60 min after injection. Microsomes were isolated and subjected to phospholipase treatment in the presence of albumin. After thin-layer chromatography of both control and treated microsomal phospholipids, the amount of phospholipid and radioactivity was determined in the individual spots. For these measurements, the iodine-stained spots were scraped off and the phospholipids were extracted with methanol (3 × 30 min at 60°C). The methanol was evaporated by heating, and the lipids were dissolved in 0.5 ml methanol. The amount of phosphate and radioactivity was measured as described previously (24). The values shown are means of four experiments.

in its quite homogeneous distribution, since it is partially inactivated by trypsin attack on both the outer and inner surface and is partially resistant to proteolytic attack from both sides. The apparent symmetric distribution of P-450 is by no means in disagreement with the concept of asymmetric distribution of membrane components. A large part of the microsomal membrane structure (5–15%) is made up of this enzyme; it is not present in stoichiometric amounts with other electron transport enzymes but is in excess, and, what is most important, it occurs in several forms (9). It is possible that the enzyme, distributed in different compartments, participates in different functions (5). On the other hand, we have no way of measuring the activity of the P-450 and we cannot exclude the possibility that the enzyme after trypsin treatment loses the capacity to be enzymatically reduced. As regards the DT-diaphorase, one portion of the enzyme is apparently tightly associated with and protected by the membrane structure and another portion is very loosely associated with the inner surface. It may well be that this diaphorase in the membrane-bound form represents newly synthesized enzyme molecules, and that the enzyme loosely bound within the channel system could represent molecules in the process of transport to their final destination, which is the soluble cytoplasm. Previous studies have shown that the large majority of this enzyme is located in nonparticulate form and recovered in the particle-free supernate after homogenization and fractionation (22).

NADH-ferricyanide reductase, the flavoprotein component of the NADH-oxidizing microsomal system, was only slightly inactivated by the proteases used in our experiments. This activity was decreased to a larger extent by combined trypsin and phospholipase treatment, which, however, may still not represent the true situation. Previous experiments with lysosomes and cathepsin D have suggested that this enzyme is probably associated with the cytoplasmic surface of the ER membrane (30, 58).

The method used to study enzyme distribution in microsomal membranes has some limitations as discussed above, and it cannot be excluded that in some cases the interpretation is uncertain. On the other hand, various types of investigations on the localization of a number of enzymes have resulted in conclusions similar to those reached in this paper. The distributions of NADH- and NADPH-cytochrome *c* reductase, cytochrome b_5, nucleoside pyrophosphatase, cytochrome P-450, G6Pase, acetanilid esterase, IDPase, and GDP-mannosyltransferase were studied by using proteolytic treatment (1, 13, 31, 34, 47); the localization of P-450 was investigated with ^{125}I-labeling (63); the localization of cytochrome b_5, NADH- and NADPH-cytochrome *c* reductases, IDPase, and acetanilide esterase was investigated by using antibodies (1, 34, 35, 46, 48, 51, 59–61); the site of various electron-transport enzymes, IDPase, G6Pase, and UDPGA-transferase, was analyzed by studying substrate permeability and latency (2, 21, 23, 27, 34); the asymmetry of esterases was studied by using charged and uncharged substrates and inhibitors (28); the site of G6Pase was investigated by histochemical methods (18, 36, 37); and, finally, the distribution of a number of electron-transport enzymes was studied by following the product released both in vitro and in vivo (25, 43). The data in the above references are generally in agreement with the findings described in this paper, which therefore confirms previous findings on the localization of some enzymes attained by using a different approach.

As with the distribution of protein species, a division of microsomal phospholipids into outer and inner compartments on the two sides of a "central line" is merely arbitrary. There is no indication at present of the factors which terminate the lipolysis of intact microsomes. Under isotonic conditions, simultaneous proteolysis does not seem to extend hydrolysis by phospholipases significantly. Therefore, the shielding of membrane phospholipids by proteins does not seem likely. There are no data concerning lipid packing in the microsomal membranes available and so we cannot assess the possible influence of lateral surface pressure on phospholipase action in this system (15). With regard to substrate specificity of the phospholipases, it is clear that this was not a limiting factor in our experiments.

The possibility of obtaining information about microsomal membranes by phospholipase treatment seems limited in comparison to the possibilities with erythrocytes. Small amounts of lysophospholipids abolish the microsomal permeability barrier to macromolecules and prevent a number of studies of membrane organization. The presence of fatty acids and lysophospholipids, generated during phospholipase treatment, may cause perturbation in the membrane, resulting in some reorganization of the phospholipids. One may also question whether P-inositol and sphingomyelin

are actually distributed at the inner face of the membrane, or whether these lipids are only buried in a manner such that the phospholipase has no access to them for steric reasons. In our experiments, phospholipase A_2 and sphingomyelinase affected P-inositol and sphingomyelin, respectively, only if the membrane was dissolved by 0.5% DOC, was rendered permeable by 0.05% DOC, or was deteriorated by phospholipid lysocompounds in the absence of albumin. Also, proteolytic pretreatment of microsomes was ineffective in increasing the enzymic hydrolysis of P-inositol or sphingomyelin which, however, were hydrolyzed when the enzymic treatment was performed in permeable vesicles. Consequently, in spite of the lack of direct experimental evidence, it appears justified to propose a tentative asymmetric distribution of phospholipids in microsomal membranes. According to this proposal, P-choline is found in both layers of the membrane bilayer. P-ethanolamine and P-serine are found mainly at the cytoplasmic surface, and P-inositol and sphingomyelin are situated chiefly at the luminal surface.

There are several possible explanations for protein and lipid asymmetry in microsomes. Considerations involving the availability of substrates and the final site of product deposition may be decisive for localization. NADPH-cytochrome *c* reductase utilizes NADPH from the cytoplasmic pool, and there is a possibility that G6Pase is part of a transferase reaction involved in the synthesis of glucose-6-phosphate at the inner microsomal surface (44). Association with a certain type of lipid may also require tighter binding to the membrane. The transverse distribution of phospholipids is opposite to that found in erythrocyte membranes (64), but it remains to be seen whether the distribution in the microsomes is also opposite to that in the liver plasma membrane. This may be the case if membrane movement and fusion with plasma membranes occur by the process of exocytosis (49). The distribution of lipid biosynthetic pathways and exchange processes seem to suggest an explanation for the asymmetric lipid distribution in erythrocytes (52, 64), and this may also be the case with microsomes. Individual phospholipids may also accumulate around asymmetrically distributed enzymes as activators (57). Finally, a relatively homogeneous lipid layer may facilitate the lateral movement of some proteins in the ER membranes during the biosynthetic process. Such a movement from rough to smooth microsomes is described for NADPH-cytochrome *c* reductase and for cytochrome b_5 (54) and may also occur in the transport of newly synthesized membrane glycoproteins (19).

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REFERENCES

1. Akao, T., and T. Omura. 1972. Acetanilide-hydrolyzing esterase of rat liver microsomes. *J. Biochem. (Tokyo)* **72:**1245–1256.
2. Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thines-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* **61:**201–212.
3. Barr, R. M., and L. M. de Luca. 1974. The in vivo incorporation of mannose, retinol and mevalonic acid into phospholipids of hamster liver. *Biochem. Biophys. Res. Commun.* **60:**355–363.
4. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, W. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* **61:**188–200.
5. Bergman, A., and G. Dallner. 1976. Properties of a rat liver smooth microsomal subfraction not aggregated by Mg^{2+}. *Life Sci.* **18:**1083–1090.
6. Bjørnstad, P. 1965. Phospholipase activity in rat-liver microsomes studied by the use of endogenous substrates. *Biochim. Biophys. Acta.* **116:**500–510.
7. Bretscher, M. S. 1972. Phosphatidyl-ethanolamine: Differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. *J. Mol. Biol.* **71:**523–528.
8. Chevallier, J., J. P. Bonnet, J. P. Tenu, and T. Gulik-Krzywicki. 1975. "Inside-out" sarcoplasmic reticulum vesicles. *FEBS (Fed. Eur. Biochem. Soc.) Proc. Meet.* **10:**1136. (Abstr.)
9. Coon, M. J., D. A. Haugen, F. P. Guengerich, J. L. Vermilion, and W. L. Dean. 1976. Liver microsomes. *In* The Structural Basis of Membrane Function. Y. Hatefi and L. Djavadi-Ohaniance, editors. Academic Press, Inc. New York. 409–427.
10. Cremona, T., and E. B. Kearney. 1963. Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. VI. Further purification and properties of the enzyme from beef heart. *J. Biol. Chem.* **239:**2328–2334.

11. DALLMAN, P. R., G. DALLNER, A. BERGSTRAND, and L. ERNSTER. 1969. Heterogeneous distribution of enzymes in submicrosomal membrane fragments. *J. Cell Biol.* **41**:357-377.
12. DALLNER, G. 1974. *Methods Enzymol.* **31**:191-201.
13. DALLNER, G., M. E. DE TOMAS, O. NILSSON, and F. W. HEMMING. 1975. Separation of pathways for synthesis of secretory and membranous glycoproteins in rough microsomes. *FEBS (Fed. Eur. Biochem. Soc.) Proc. Meet.* **10**:1096. (Abstr.)
14. DALLNER, G., P. SIEKEVITZ, and G. E. PALADE. 1966. Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. *J. Cell Biol.* **30**:73-96.
15. DEMEL, R. A., W. S. M. GEURTS VAN KESSEL, R. F. A. ZWAAL, B. ROELOFSEN and L. L. M. VAN DEENEN. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. *Biochim. Biophys. Acta.* **406**:97-107.
16. DEPIERRE, J. W., and G. DALLNER. 1975. Structural aspects of the membrane of the endoplasmic reticulum. *Biochim. Biophys. Acta* **415**:411-472.
17. DEPIERRE, J. W., and M. L. KARNOVSKY. 1973. Plasma membranes of mammalian cells. *J. Cell Biol.* **56**:275-303.
18. EL-AASER, A. A., J. T. R. FITZSIMMONS, R. H. HINTON, K. A. NORRIS, and E. REID. 1973. Electron microscopic examination of hepatic subcellular fractions for phosphatases. *Histochem. J.* **5**:199-223.
19. ELHAMMER, Å., H. SVENSSON, F. AUTUORI, and G. DALLNER. 1975. Biogenesis of microsomal membrane glycoproteins in rat liver. III. Release of glycoproteins from the Golgi fraction and their transfer to microsomal membranes. *J. Cell Biol.* **67**:715-724.
20. ERIKSSON, L. C. 1973. Studies on the biogenesis of endoplasmic reticulum in the liver cell. *Acta Pathol. Microbiol. Scand. Suppl.* **239**:1-72.
21. ERNSTER, L. 1958. Structural factors involved in the diaphorase and cytochrome *c*-reductase activities of mitochondria and microsomes. *Acta Chem. Scand.* **12**:600-602.
22. ERNSTER, L., L. DANIELSON, and M. LJUNGGREN. 1962. DT diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. *Biochim. Biophys. Acta.* **58**:171-188.
23. ERNSTER, L., and L. C. JONES. 1962. A study of the nucleoside tri- and diphosphatase activities of rat liver microsomes. *J. Cell Biol.* **15**:563-578.
24. GLAUMANN, H., and G. DALLNER. 1968. Lipid composition and turnover of rough and smooth microsomal membranes of rat liver. *J. Lipid Res.* **9**:720-729.
25. GLAUMANN, H., R. NILSSON, anmd G. DALLNER. 1970. Cytoplasmic location of certain small molecules after their metabolism by the endoplasmic reticulum of rat liver. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **10**:306-308.
26. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**:751-766.
27. GRATZL, M. 1975. Membrane effects on hepatic microsomal glucose-6-phosphatase. *Hoppe-Seyler's Z. Physiol. Chem.* **356**:861-865.
28. GRATZL, M., W. NASTAINCZYK, and D. SCHWAB. 1975. The spatial arrangement of esterases in the microsomal membrane. *Cytobiologie.* **11**:123-132.
29. HARMON, H. J., J. D. HALL, and F. L. CRANE. 1974. Structure of mitochondrial christae membranes. *Biochim. Biophys. Acta.* **344**:119-155.
30. ITO, A. 1973. Evidence obtained by cathepsin digestion of microsomes for the assembly of cytochrome b_5 and its reductase in the membrane. *J. Biochem. (Tokyo)* **75**:787-793.
31. ITO, A., and R. SATO. 1969. Proteolytic microdissection of smooth-surfaced vesicles of liver microsomes. *J. Cell Biol.* **40**:179-189.
32. JACKSON, R. L., J. P. SEGREST, I. KAHANE, and V. T. MARCHESI. 1973. Studies on the major sialoglycoprotein of the red cell membrane. *Biochemistry.* **12**:3131-3138.
33. KREIBICH, G., P. DEBEY, and D. D. SABATINI. 1973. Selective release of content from microsomal vesicles without membrane disassembly. *J. Cell Biol.* **58**:436-462.
34. KURIYAMA, Y. 1972. Studies on microsomal nucleoside diphosphatase of rat hepatocytes. *J. Biol. Chem.* **247**:2979-2988.
35. KURIYAMA, Y., T. OMURA, P. SIEKEVITZ, and G. E. PALADE. 1969. Effects of phenobarbital on the synthesis and degradation of the protein components of rat liver microsomal membranes. *J. Biol. Chem.* **244**:2017-2026.
36. LESKES, A., P. SIEKEVITZ, and G. E. PALADE. 1971. Differentiation of endoplasmic reticulum in hepatocytes. I. Glucose-6-phosphatase distribution in situ. *J. Cell Biol.* **49**:264-287.
37. LEWIS, J. A., and J. R. TATA. 1973. Heterogeneous distribution of glucose-6-phosphatase in rat liver microsomal fractions as shown by adaptation of a cytochemical technique. *Biochem. J.* **134**:69-78.
38. LODISH, H. F., and B. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. *J. Cell Biol.* **65**:51-64.
39. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
40. MARINETTI, G. V. 1962. Chromatographic separation, identification and analysis of phosphatides. *J. Lipid Res.* **3**:1-20.
41. MARINETTI, G. V. 1965. Chromatography of lipids

on commercial silica gel loaded filter paper. *J. Lipid Res.* **6**:315-317.
42. NILSSON, O., and G. DALLNER. 1975. Distribution of constitutive enzymes and phospholipids in microsomal membranes of rat liver. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **58**:190-193.
43. NILSSON, R., E. PETERSON, and G. DALLNER. 1973. Permeability of microsomal membranes isolated from rat liver. *J. Cell Biol.* **56**:762-776.
44. NORDLIE, R. C. 1974. Metabolic regulation by multifunctional glucose-6-phosphatase. *Curr. Top. Cell. Regul.* **8**:33-117.
45. OJAKIAN, G., G. KREIBICH, J. KRUPPA, D. MOK, and D. D. SABATINI. 1975. Lateral movement of ribosomes on microsomal membranes detected by freeze etching. *Fed. Proc.* **34**:536.
46. OMURA, T., Y. KURIYAMA, S. TAKESUE, and N. OSHINO. 1970. Microsomes and the drug-oxidising enzyme system. *Int. Congr. Biochem. Abstr.* **8**:260. (Abstr.)
47. ORRENIUS, S., A. BERG, and L. ERNSTER. 1969. Effects of trypsin on the electron transport system of liver microsomes. *Eur. J. Biochem.* **11**:193-200.
48. OSHINO, N., and T. OMURA. 1973. Immunochemical evidence for the participation of cytochrome b_5 in microsomal stearyl-CoA desaturation reaction. *Arch. Biochem. Biophys.* **157**:395-404.
49. PALADE, G. E. 1973. Interactions among cellular membranes. *Int. Congr. Biochem. Abstr.* **9**:7. (Abstr.)
50. PARKER, F., and N. F. PETERSON. 1965. Quantitative analysis of phospholipids and phospholipid fatty acids from silica gel thin-layer chromatograms. *J. Lipid Res.* **6**:455-459.
51. REMACLE, J., S. FOWLER, H. BEAUFAY, and J. BERTHET. 1974. Ultrastructural localization of cytochrome b_5 on rat liver microsomes by means of hybrid antibodies labeled with ferritin. *J. Cell Biol.* **61**:237-240.
52. RENOOIJ, W., L. M. G. VAN GOLDE, R. F. A. ZWAAL, B. ROELOFSEN, and L. L. M. VAN DEENEN. 1974. Preferential incorporation of fatty acids at the inside of human erythrocyte membranes. *Biochim. Biophys. Acta.* **363**:287-292.
53. SIEKEVITZ, P. 1963. Protoplasm: endoplasmic reticulum and microsomes and their properties. *Annu. Rev. Physiol.* **25**:15-37.
54. SIEKEVITZ, P., G. E. PALADE, G. DALLNER, I. OHAD, and T. OMURA. 1967. The biogenesis of intracellular membranes. *In* Organizational Biosynthesis. H. J. Vogel, J. O. Lampen, and V. Bryson, editors. Academic Press, New York 331-362.
55. SPATZ, L., and P. STRITTMATTER. 1971. A form of cytochrome b_5 that contains an additional hydrophobic sequence of 40 amino acid residues. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1042-1046.
56. STECK, T. L. 1974. The organization of proteins in the human red blood cell membrane. *J. Cell Biol.* **62**:1-19.
57. STIER, A., and E. SACKMANN. 1973. Heterogeneous lipid distribution in liver microsomal membranes. *Biochim. Biophys. Acta.* **311**:400-408.
58. TAKESUE, S., and T. OMURA. 1968. Enzymatic solubilization of microsomal NADH-cytochrome b_5 reductase by lysosomes. *Biochem. Biophys. Res. Commun.* **30**:723-729.
59. TAKESUE, S., and T. OMURA. 1970. Solubilization of NADH-cytochrome b_5 reductase from liver microsomes by lysosomal digestion. *J. Biochem. (Tokyo).* **67**:259-266.
60. TAKESUE, S., and T. OMURA. 1970. Purification and properties of NADH-cytochrome b_5 reductase solubilized by lysosomes from rat liver microsomes. *J. Biochem. (Tokyo).* **67**:267-276.
61. TAKESUE, S., and T. OMURA. 1970. Immunological similarity between NADH-cytochrome *c* reductase of mitochondrial outer membrane and microsomes. *Biochem. Biophys. Res. Commun.* **40**:396-401.
62. VERKLEIJ, A. J., R. F. A. ZWAAL, B. ROELOFSEN, P. COMFURIUS, D. KASTELIJN, and L. L. M. VAN DEENEN. 1973. The asymmetric distribution of phospholipids in the human red cell membrane. *Biochim. Biophys. Acta.* **323**:178-193.
63. WELTON, A. F., and S. D. AUST. 1974. The effects of 3-methyl-cholanthrene and phenobarbital induction on the structure of rat liver endoplasmic reticulum. *Biochim. Biophys. Acta.* **373**:197-210.
64. ZWAAL, R. F. A., B. ROELOFSEN, and C. M. COLLEY. 1973. Localization of red cell membrane constituents. *Biochim. Biophys. Acta.* **300**:159-182.