PERMEABILITY OF MICROSOMAL MEMBRANES ISOLATED FROM RAT LIVER

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ABSTRACT

Water compartments, permeability, and the possible active translocation of various substances in rat liver microsomes were studied by using radioactive compounds and ultracentrifugation. The total water of the microsomal pellet, 3.4 μl/mg dry weight, is the sum of water in the extramicrosomal and intramicrosomal spaces, or 56 and 44%, respectively. Sucrose space accounts for 77% of the intramicrosomal water and the hydration water $\sim$ 14%, leaving almost no sucrose-impermeable space when using the ultracentrifugation approach. With increasing sucrose concentration, microsomes do not show an osmotic response. The intramicrosomal water decreases greatly in the presence of Cs^+ and Mg^{++} in rough but not in smooth microsomes. Uncharged substances of molecular weight of up to at least 600 freely penetrate microsomal membranes, which already become impermeable to charged substances at a molecular weight of 90. These substances also induce an osmotic response. The vesicles can be made permeable to charged substances after water treatment and cooling, which, however, does not increase glucose-6-phosphatase and inosine diphosphatase (IDPase) activities, and these enzymes can still be activated by deoxycholate. IDPase, reduced nicotinamide adenine dinucleotide-cytochrome c reductase, and reduced nicotinamide adenine dinucleotide phosphate-dependent hydroxylation reactions, performed in vitro, also disproved the hypothesis of an accumulation of charged substances inside of vesicles of being a major pathway. The products of the enzymic reactions as well as the glucuronidated form of a hydroxylated product can be recovered on the cytoplasmic side of membranes, and little accumulation occurs in the intravesicular compartment.

INTRODUCTION

Various experiments have indicated that microsomal membranes are semipermeable (1–5). Such semipermeability may have important functional implications, since permeability barriers may affect the accessibility of enzymes located in these organelles to their natural substrates. It is conceivable that one important aspect of membrane function is to regulate the activity of enzymic systems by varying membrane permeability, as exemplified by the effect of insulin on the penetration of glucose and amino acids through the plasma membrane (6, 7). No such example has yet been demonstrated in the case of microsomes, although the latency of enzymes such as glucose-6-phosphatase (G6Pase)[1] (8), inosine diphosphatase (IDPase)

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[1] *Abbreviations used in this paper:* ATP, adenosine triphosphate; DOC, deoxycholate; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; IDP, inosine diphosphate; IMP, inosine monophosphate; NAD,

(9), and uridine diphosphoglucuronic acid (UD-PGA) transferase (10) indicates the presence of a membrane barrier.

Differences in permeability and size may be basic physicochemical factors which have permitted the separation of microsomal vesicles into submicrosomal fractions exhibiting different enzymic composition (cf. reference 11). If the microsomal membrane is not permeable to the solute used for building up the gradient, the osmotic response of the vesicles will affect the sedimentation behavior of the particles during centrifugation (12, 13).

The liver endoplasmic reticulum (ER) plays a central role in the synthesis of plasma proteins (14, 15), various types of lipoproteins (16-18), cholesterol (19, 20), and glycoproteins (21, 22), and in the metabolism of small molecules of physiological as well as nonphysiological origin (cf. reference 23). The newly synthesized macromolecules follow the pathway rough ER → smooth ER → Golgi system, where a concentration occurs before there is release into the blood (24-30). The fate of the metabolic products of small molecules is not clarified, however. It has been repeatedly suggested that the products of the metabolism of small molecules also follow the same pathway by, for instance, the involvement of translocators. Recent experiments indicated, however, that products of enzymic hydroxylation of a hydrocarbon in vivo were released into the cytoplasm (31).

A further aspect of permeability is the relationship which seems to exist between membrane permeability and the kinetic properties of a bound enzyme (cf. reference 32). In a number of experimental pathological conditions, microsomal functions display profound change, which appears primarily as membrane dysfunction rather than a direct enzyme effect. Such changes occur in the initial phase of alloxan diabetes (33), in which the V_{max} and K_m of G6Pase increase, as well as during enzymic lipid peroxidation (34, 35), which results in activation of IDPase, activation-inhibition of nicotinamide

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nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; P_i, inorganic orthophosphate; PLP, phospholipid; PP_i, inorganic pyrophosphate; UDPGA, uridine diphosphoglucuronic acid.

adenine dinucleotide, reduced form (NADH)-cytochrome c reductase and G6Pase, and inactivation of adenosine triphosphatase (ATPase).

In this paper, the permeability of microsomal membranes towards charged and uncharged molecules is investigated together with the distribution of reaction products derived from the metabolism of small molecules. Preliminary reports of this work have appeared elsewhere (36, 37).

MATERIALS AND METHODS

Animals

Adult male albino rats weighing 180-200 g were used. The animals were fasted for 20 h before sacrifice unless otherwise stated.

Fractionations

Total microsomes from rat liver were prepared according to Ernster et al. (38). The microsomal pellets were suspended in 0.5 M sucrose when routine experiments were made for determination of water compartments. In the experiments in which the suspending media were other than 0.5 M sucrose, indication is given in the appropriate table or figure.

Rough and smooth microsomes were prepared according to Rothschild (39), with the modifications described previously (40). Both subfractions were suspended in 0.5 M sucrose.

H_2O Treatment

In order to test the permeability of microsomal vesicles, the pellet was resuspended in distilled water and processed principally according to Schramm et al. (41). The concentration was 1 g/20 ml water. This suspension was incubated in a water bath at 30°C for 15 min. The suspension in the large Erlenmeyer flask was placed thereafter into an ice-water bath. This treatment is effective in removing the content of the vesicles. The cold suspension was centrifuged at 105,000 g for 60 min and the pellet was resuspended in 0.25 or 0.5 M sucrose according to the experiment.

Millipore Filtration

At the end of incubation in the various enzyme reactions, the medium was separated from the particulate material by Millipore filtration as in previous studies (42). The suspensions were filtered by suction pump through a Millipore filter with a size of 0.22 μm (Millipore Corp., Bedford, Mass.). No additional washing was applied after filtration. Control solutions were passed through the filter to estimate the adsorp-

tion by the filter of the various charged substances. This adsorption was about 10% or less, and the values were used for corrections.

Dialysis

For dialysis, 10 ml of suspensions containing microsomes were placed in the dialysis bag and dialyzed 18 h under continuous stirring in a cold room. The dialysis medium was changed 8-10 times on each occasion.

Calculation of Water Compartments

Freshly prepared microsomes were suspended, in previously weighed tubes, in an incubation medium containing the labeled solute and an appropriate amount of carrier in 0.25 M sucrose. After centrifugation for 2 h at 105,000 g, the supernate was decanted. The insides of the tubes were carefully wiped off with filter paper and the pellets (approximate volume 0.3 ml) resuspended in 3 ml of formic acid. The tubes were then reweighed and the density of the suspension was accurately determined from a sample to obtain the exact total volume. 0.2 ml of the formic acid solution was added to 10 ml Bray's solution (43) and the activity determined by liquid scintillation counting.

For determination of the activity of 1 ml of the medium containing the labeled compound in homogeneous solution, separate standard measurements were made in solutions of equal volume in the absence of microsomes but with the same proportion of formic acid added before counting. The total accessible space to the solute was then calculated according to

$$\text{solute accessible space} = \frac{\text{total cpm in pellet}}{\text{cpm/ml in homogeneous solution}}.$$

The total water was estimated by measuring the wet weight-dry weight differences of pellets dried over H_2SO_4 at 80°C. Constancy of the values was ensured by repeated weighings during 3 days of evaporation. The amount of sucrose occluded in the pellet was determined from the sucrose-accessible space of the pellet in each experiment and the weight of the sucrose was then subtracted from the dry weight. The total water space of the pellet was likewise corrected by adding the volume occupied by the sucrose.

THE EXTRAMICROSOMAL SPACE: The extramicrosomal space, i.e. the water present in the space between the vesicles, was determined by using ^{14}C-labeled carboxydextran (mol wt 10,000) in the presence of unlabeled carrier with a concentration of 15 mg/ml. By conductivity measurements, the unlabeled dextran was found to contain considerable amounts of ionic substances, which were removed by extensive

dialysis before use. Ions may cause aggregation of the microsomes, with the result that artifacts may occur. When estimating the accessible space for various solutes, the dextran space (extramicrosomal space) was also measured in the presence of the unlabeled solutes in question.

INTRAMICROSOMAL WATER: Intramicrosomal water was obtained by subtracting the extramicrosomal water (dextran space) from the total water content of the pellet. The accessible microsomal space of other solutes was likewise obtained by subtracting the extramicrosomal water from the total space occupied by the solute in the pellet.

HYDRATION OF THE MATRIX: The hydration of the matrix was determined by comparing the median equilibrium density of total microsomes in a sucrose gradient using H_2O as a solvent with a sucrose gradient made up by D_2O (44, 45). From the two apparent densities of the microsomes found after isopycnic centrifugation, the approximate amount of hydration water exchangeable with D_2O could be determined by using the expression

$$\frac{V_m \times \rho_m + V_w \times \rho_w}{V_m + V_w} = \rho_{tot},$$

for the median equilibrium densities in H_2O and D_2O. V_m is the volume of the matrix, V_w volume of the hydration water, ρ_m the density of the matrix, ρ_w the density of the hydration water (D_2O), and ρ_{tot} the total density of the particle.

Chemical and Radioactivity Analysis

The radioactive substances were purchased from The Radiochemical Centre, Amersham, England, or from the New England Nuclear Corp., Boston, Mass. Glycerol, ouabain, acetate, mevalonate, and IDP were ^3H-labeled, and dextran, glucose, sucrose, maltotriose, inulin, G6P, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), morphine, naphthalene, and naphthol were ^{14}C-labeled. Radioactive NAD was reduced enzymatically with alcohol dehydrogenase (46), and NADP was reduced with isocitrate dehydrogenase (47).

In all measurements, regardless of whether the suspended pellet, supernate, or standard was used, an identical amount of formic acid was added. Bray's solution was used as scintillator (43), and radioactivity was measured in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Protein was determined according to Lowry et al. (48) with bovine serum albumin as standard. Phospholipid (PLP) was measured as described previously (49).

Enzyme Assays

The activities of IDPase and G6Pase were determined as before (50). In the experiments in which product localization was studied, inorganic orthophosphate (P_i), both in the supernate and in the pellet, was measured according to Lindberg and Ernster (51). For the estimation of reduced cytochrome c, a portion of the supernate was reduced with dithionite and the increment at 550 nm gave the oxidized cytochrome c left after the incubation. The difference between this value and the amount originally present in the incubation medium gave the value for cytochrome c reduced during the incubation. The formaldehyde formed during morphine hydroxylation was estimated by the Nash reaction (52).

When hydroxylation of naphthalene was studied, the reaction was stopped by Millipore filtration. Solid polyethylene has previously been found to act as an extremely efficient extraction medium for nonpolar hydrocarbons present in saturated aqueous solutions (53). By shaking a saturated solution of pure naphthalene in a polyethylene vial, the hydrocarbon is removed quantitatively from the solution. Phenols such as the products of enzymic hydroxylation of naphthalene are not adsorbed by the plastic, which permits an accurate determination of polar products formed from labeled naphthalene after removal of the unchanged hydrocarbon.

RESULTS

Methodological Aspects

Since the volume of the apparent extramicrosomal space was determined by using [^{14}C]carboxydextran of approximate molecular weight of 10,000, errors might be introduced by the partial sedimentation of the high molecular weight compound. In Fig. 1, the effect of centrifugation time on sedimentation was measured. No sedimentation could be detected after 2 h. The extramicrosomal space was almost exactly identical with the extramicrosomal water space obtained by using labeled compounds of low molecular weight, such as acetate and mevalonate, which did not penetrate the microsomal membrane. The absence of any contamination of the labeled dextran by radioactive compounds of low molecular weight was checked by dialysis.

Apart from the fact that the extramicrosomal space diminished somewhat when using microsomes from nonstarved rats, no significant differences could be detected between starved and nonstarved rats in the apparent penetration of various solutes. However, because of the lower
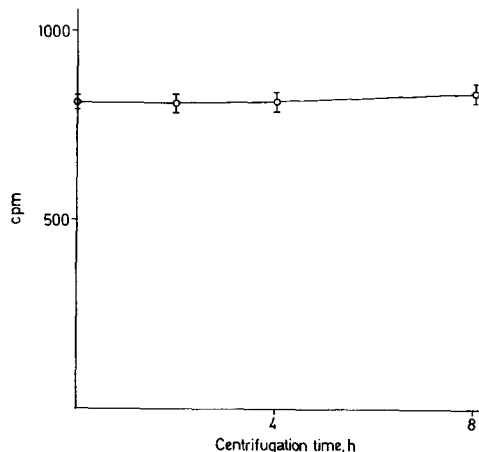

FIGURE 1 Lack of sedimentation of [^{14}C]carboxydextran during ultracentrifugation. One experimental series consisted of four centrifuge tubes containing 0.25 M sucrose, 15 mg/ml unlabeled dextran, and 40 μl [^{14}C]carboxydextran (5 μCi/ml). After varying periods of centrifugation, the tubes were punctured at the bottom and only the first 0.3 ml fraction, i.e. the fraction at the bottom, was removed. 0.05 ml of this fraction were added to 10 ml Bray's solution (43) and the radioactivity was measured. The results represent the means of five experiments.

recovery of microsomes and possible complications arising from the presence of large amounts of glycogen, starved rats were used in all experiments.

The most serious difficulty in experiments of this type is to distinguish between a true penetration of a solute and increased radioactivity in the pelleted microsomes due to adsorption. As seen from Table I, the adding of unlabeled dextran in amounts up to 30 mg of dextran per ml of the suspending medium decreased the total radioactivity in the pellet by approximately 15%. Higher dextran concentrations were found to cause incomplete sedimentation of the microsomes because of the marked increase in the total density of the medium. In our experiments, the amount of unlabeled dextran added was 15 mg.

A certain adsorption was consistently observed for all the substances studied. The addition of unlabeled carrier in amounts between 0.01 and 0.1 M (depending on the substance in question) counteracted this adsorption effect for noncharged water-soluble substances (Fig. 2). As for charged substances, on the other hand, adsorption could only be suppressed by the addition of unlabeled

carrier in a few instances like acetate, mevalonate, and G6P. (Demonstrated for mevalonate in Fig. 3.) This limited our choice, since for substances like choline, leucine, NAD, NADP,

IDP, inorganic pyrophosphate (PP$_i$), and P$_i$, an unreasonably high radioactivity was found in the pellet, which could not be accounted for even by total penetration.

Water Compartments

The total microsomal water present in the pelleted material is distributed in an extramicrosomal and an intramicrosomal water space (Table II). The intramicrosomal water com-

TABLE I

Effect of Carrier Dextran on the Amount of [^{14}C]Carboxydextran in the Microsomal Pellet

Carrier	cpm in pellet	Total H₂O in pellet
mg dextran/ml		*µl/mg dry weight*
None	618	2.45
5	539	2.44
15	517	2.43
20	520	2.44
30	522	2.42

Microsomes were suspended in 0.5 M sucrose in a concentration which corresponds to about 4 g wet weight of liver/10 ml. 5.5 ml of this suspension was placed in a Spinco 40 centrifuge tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and either 5.4 ml cold distilled water or 5.4 ml carrier dextran solution were added in a concentration of 10, 30, 40, or 60 mg dextran/ml. Finally, all centrifuge tubes were supplemented with 0.1 ml [^{14}C]carboxydextran (5 µCi/ml). After mixing the contents by turning the tubes upside down six times, centrifugation, and further processing were performed as described in Materials and Methods. The pellet was suspended in 3 ml formic acid. 0.2 ml was taken from this suspension and used for the determination of radioactivity.

TABLE II

Water Compartments of Liver Microsomal Pellet

Water compartment	µl/mg dry weight	% of total	% of intramicrosomal water
Total microsomal water	3.36 ± 0.09	100	
Extramicrosomal water	1.88 ± 0.06	56	
Intramicrosomal water	1.48 ± 0.06	44	100
Sucrose space	1.14 ± 0.07		77
Sucrose-inaccessible space and hydration water	0.34 ± 0.03		23

All the measurements were made as described in Materials and Methods. The values represent the means of 13 experiments ±SEM.
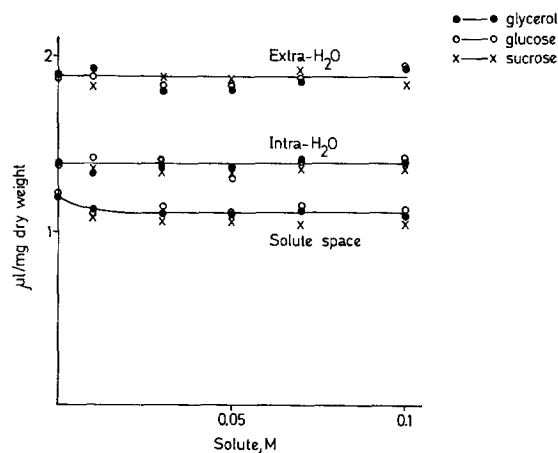

FIGURE 2 Effect of glycerol, glucose, and sucrose concentrations on the microsomal water compartments. The intramicrosomal water was measured as described in Materials and Methods. The glycerol, glucose, and sucrose concentrations of the individual tubes are given on the abscissa.

partment was found to be 1.5 μl H₂O/mg of dry weight and includes a sucrose-accessible space amounting to 1.1 μl/mg dry weight. By determining the mean equilibrium density of microsomes in H₂O and D₂O according to Beaufay et al. (44, 45), the amount of hydration water could be estimated to be about 0.2 μl/mg dry weight. Evidently, the amount of water that could therefore be involved in osmotic shifts is very small compared with the total water content of the vesicles.

Cations had an appreciable effect on both the extra- and the intramicrosomal water spaces (Table III). The total microsomal water decreased to a certain extent in the presence of Cs⁺ and to an even greater degree in the presence of Mg²⁺. This effect is mainly due to a decrease in the extramicrosomal water and reflects

TABLE III

Effect of Cs⁺ and Mg⁺⁺ on Water Compartments of Rough and Smooth Microsomal Pellets

Fraction	Cation	Total water	Extrami- crosomal water	Intrami- crosomal water
		μl per mg dry weight		
Total microsomes	none	3.28	1.84	1.44
Rough microsomes	none	3.37	1.65	1.72
Smooth microsomes	none	3.18	1.90	1.28
Total microsomes	Cs⁺	2.76	1.59	1.17
Rough microsomes	Cs⁺	2.20	1.33	0.87
Smooth microsomes	Cs⁺	2.68	1.52	1.16
		Γ		
Total microsomes	Mg⁺⁺	2.15	1.01	1.14
Rough microsomes	Mg⁺⁺	1.89	1.17	0.72
Smooth microsomes	Mg⁺⁺	2.32	1.14	1.18

Rough and smooth microsomes were prepared by modification of Rothschild's procedure. In the experiments containing cations, the final concentration of CsCl was 15 mM and of MgCl₂ 10 mM. The experiments were conducted as those in Table II. The values represent the means of three experiments.

an aggregation of the vesicles resulting in a tighter packing. Isolated rough and smooth vesicles behaved very much the same as regards the extramicrosomal water. On the other hand, the intramicrosomal water compartment of the two subfractions showed a different response toward the two cations. Thus, the rough microsomes contracted far more in the presence of both Cs⁺ and Mg²⁺ than their smooth counterparts.

Apparent Permeability

Noncharged substances with molecular weights ranging from 80 to 580 appeared to penetrate the microsomal membrane more or less freely within the time of centrifugation employed (Table IV). The upper limit of the size of penetrable uncharged substances could not be accurately determined because of difficulties in finding suitable, water-soluble test substances. However, the limit is certainly lower than 5,000 to judge from the results obtained with inulin.

Charged substances, with as low a molecular weight as that of acetate, did not penetrate the membrane. A certain degree of penetration was observed in a few cases with G6P, but these results were variable. Hydrolytic reactions may, in these cases, have split some of the sugar phosphate into inorganic phosphate and glucose. The glucose, carrying the label, thus readily penetrates the membrane.

It would appear, thus, that microsomal membranes are penetrable to uncharged molecules of up to a relatively high molecular weight but not to negatively charged substances like acetate or mevalonate.

Nature of the "Sucrose Space"

The results shown in Table IV indicate that an "osmotic space" is virtually absent when liver microsomes are suspended in 0.25 M sucrose. In order to investigate this problem further, the influence of glycerol, glucose, and sucrose on the size of the microsomal vesicle as given by the intramicrosomal water space was studied over a broader concentration range (Fig. 2). Clearly, the amount of intramicrosomal water is not appreciably affected by changes in the solute concentration within the range 0.01–0.1 M, confirming a lack of osmotic response towards these solutes. Some unspecific adsorption

TABLE IV

Apparent Penetration of Various Substances through the Microsomal Membranes

Substance	Extramicrosomal H_2O space	Intramicrosomal H_2O space	Microsomal substance space	Substance space / Intramicrosomal space × 100
	µl per mg dry weight			
–	1.88	1.48	–	
Glycerol	1.80	1.41	1.09	77
Glucose	1.83	1.38	1.10	80
Sucrose	1.85	1.38	1.06	77
Maltotriose	1.93	1.41	0.96	68
Ouabain	1.78	1.43	1.22	85
Inulin	1.91	1.51	0.05	3
Acetate	1.75	1.19	−0.04	−3
Mevalonate	1.76	1.23	0.04	3
G6P	1.71	1.20	0–0.36	0–30

Every centrifuge tube contained 5.5 ml microsomal suspension (microsomes isolated from about 2 g liver) and the following carrier solutions in the appropriate case: 1 ml 0.5 M glycerol, glucose, maltotriose, Na-acetate, mevalonate, G6P, 5.4 ml saturated ouabain solution. Water was added to all tubes (except in the case of inulin) to a final volume of 10.9 ml. Finally, 0.1 ml of appropriate radioactive substance was added (50 µCi/ml of ^3H- and 5 µCi/ml of ^{14}C-labeled compounds). Because of its low solubility and low specific activity (1 µCi/mg), the amount of radioactive inulin added was 5.5 ml; this solution contained 0.17 µCi/ml. Extra- and intramicrosomal water spaces were measured in the presence of unlabeled carrier substances. The results are the means of 6–10 experiments with the exception of maltotriose, which is based on 2 experiments.

of glucose and glycerol occurs in the absence of carrier. However, even a low amount of carrier counteracts this effect.

With a charged substance, such as mevalonate, a marked osmotic response was found (Fig. 3). The decrease in extramicrosomal water observed may be attributed to the closer packing of the vesicles caused by the cation present (see Table III). Identical results were obtained with acetate.

Theoretically, the penetration of a compound like sucrose could be only apparent and due to an unspecific adsorption of sucrose to the pelleted material. Another explanation might be that the vesicles were damaged during centrifugation.
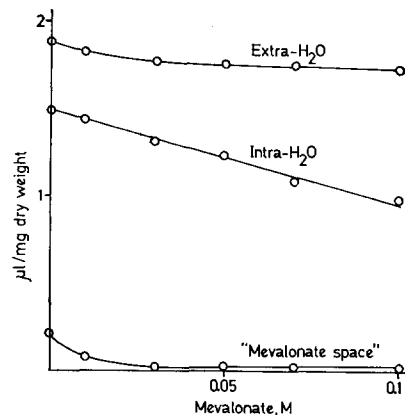
In order to investigate irreversible adsorption

FIGURE 3 Effect of mevalonate concentration on the microsomal water compartments. The intramicrosomal water was measured as described in Materials and Methods. The concentration of mevalonate in each tube is given on the abscissa.

effects, microsomes were dialyzed after incubation with labeled sucrose in the presence of varying concentrations of carrier. After 18 h dialysis, only negligible amounts of sucrose were retained by the microsomal material (Table V). The results were the same regardless of whether the microsomes were or were not sedimented by centrifugation after incubation with [^{14}C]sucrose. Identical results were also obtained when the labeled sucrose was added during homogenization with subsequent dialysis of the 10,000 g supernate, or when the label was added to the 10,000 g supernate immediately before dialysis.

The results so far obtained seem to indicate that the sucrose penetration observed cannot be an artifact. Vesicle volume is clearly independent of the sucrose concentration (Fig. 2). Further, the sucrose space is unaffected by increasing the amount of unlabeled carrier up to 1 M, which makes it highly improbable that an adsorption effect distorts the results. As evidenced by the dialysis experiment, no tight binding of sucrose occurs.

If the vesicles were damaged during centrifugation, resulting in a penetration of sucrose, it could be expected that the proportion of damaged vesicles would increase with centrifugation time. No such increase in the sucrose content of pellets obtained after various centrifugation times could be observed, however, as demonstrated by the constancy of radioactivity

Table V

Dialysis of Intramicrosomal [^{14}C]Sucrose

	Suspending medium	Dialysis medium	0 h	18 h
			cpm	
(1) Microsomes, pelleted with [^{14}C]sucrose	0.05 M sucrose	0.05 M sucrose	1,045	20
Microsomes, pelleted with [^{14}C]sucrose	0.25 M sucrose	0.25 M sucrose	943	30
Microsomes, pelleted with [^{14}C]sucrose	0.5 M sucrose	0.5 M sucrose	1,182	24
(2) Microsomes	0.25 M + [^{14}C]sucrose	0.25 M sucrose	1,224	40
(3) 10,000 g supernate	0.25 M + [^{14}C]sucrose	0.25 M sucrose	14,792	225
(4) 10,000 g supernate of homogenate with [^{14}C]sucrose	0.25 M sucrose	0.25 M sucrose	16,521	281
(5) Microsomes + [^{14}C]sucrose, centrifuged 30 min	0.25 M sucrose	0.25 M sucrose	970	15
Microsomes + [^{14}C]sucrose, centrifuged 60 min	0.25 M sucrose	0.25 M sucrose	1,051	20
Microsomes + [^{14}C]sucrose, centrifuged 120 min	0.25 M sucrose	0.25 M sucrose	890	16
Microsomes + [^{14}C]sucrose, centrifuged 180 min	0.25 M sucrose	0.25 M sucrose	820	16

Exp. 1. Livers were homogenized in 0.05, 0.25, and 0.5 M sucrose. The large particles were removed by centrifugation at 10,000 g for 20 min. The microsomes were sedimented at 105,000 g for 60 min and suspended in 0.1, 0.5, and 1.0 M sucrose (4 g/10 ml). 5.5 ml of each suspension was supplied with 5.3 ml water and 0.2 ml [^{14}C]sucrose (5 μCi/ml). Therefore, the final sucrose concentrations were 0.05, 0.25, and 0.5 M, respectively. The pellet was suspended and dialyzed as shown in the table. *Exp. 2.* Microsomes from 2 g liver were suspended in 10 ml 0.25 M sucrose, supplemented with 0.2 ml [^{14}C]sucrose (0.5 μCi/ml), and dialyzed as described in Materials and Methods. *Exp. 3.* 2 g liver was homogenized in 0.25 M sucrose and centrifuged at 10,000 g for 20 min. The supernate, "10,000 g supernate," was adjusted to 10 ml, 0.2 ml [^{14}C]sucrose (5 μCi/ml) was added, and the suspension was transferred to a dialysis bag. *Exp. 4.* 2 g liver was homogenized in 0.25 M sucrose and 0.2 ml [^{14}C]sucrose (5 μCi/ml). After centrifugation at 10,000 g for 20 min, the supernate was decanted, adjusted to 10 ml with 0.25 M sucrose, and was transferred to a dialysis bag. *Exp. 5.* The centrifuge tubes were made up as in exp. 1, but all in 0.25 M sucrose. The individual tubes were centrifuged for 30, 60, 120, and 180 min. The pellets were suspended in 0.25 M sucrose and dialyzed.

within the dialysis bag at zero time (Table V, exp. 5).

Experimentally Induced Changes in Permeability

Incubation in hypotonic media at 30°C followed by cooling has successfully been employed by Schramm et al. (41) to extract luminal protein of the salivary gland zymogen granules. This procedure has been found effective for removal of the microsomal vesicle contents (54). Electron microscope analysis of the vesicles after this type of treatment failed to reveal any significant visible rupture of the membranes.[2] As shown in Table VI, water treatment induces an increase in membrane permeability toward the charged substances acetate and mevalonate. The results are the same, though less clear, with G6P, for the possible reasons already discussed.

Membrane-bound IDPase and G6Pase are activated by deoxycholate (DOC) (8, 9). This has been interpreted as a breakdown of barriers preventing the substrates from reaching the

[2] A. Bergstrand. Personal communication.

active sites of these enzymes, and the possibility was also raised the IDPase is localized inside the vesicle. However, the DOC effect is still present after water treatment, which increases the permeability of the microsomal membrane toward charged substances as shown above (Fig. 4). From these experiments, it seems less likely that DOC activates simply by facilitating the penetration of substrate through the microsomal membrane.

Localization of Metabolic Products

The distribution pattern of metabolic products formed during enzymatic processes could be dif-

Table VI
Permeability of H_2O-Treated Microsomal Membranes

	Intramicrosomal space			
	Control		H_2O treated	
Substance	$\mu l/mg$ dry weight	% of intra-micro-somal H_2O	$\mu l/mg$ dry weight	% of intra-micro-somal H_2O
None	1.45	100	0.90	100
Acetate	0.05	3	0.68	76
Mevalonate	0.06	4	0.77	85
G6P	0-0.39	0-30	0.46-0.69	50-75

H_2O treatment and determinations of intramicrosomal space are described in Materials and Methods. The results are the means of four experiments.

ferent from that obtained when studying passive transport, and this possibility must be taken into consideration. By such processes, charged products, which passively cannot penetrate the microsomal membrane, could also accumulate inside the vesicles. With the purpose of looking for such a mechanism, the distribution of all metabolic products during the operation of IDP-ase, NADH-cytochrome c reductase, and the detoxication enzymes were studied, using, in principle, a similar approach as in a previous investigation (31).

When investigating the distribution of the substances IDP, inosine monophosphate (IMP), NAD, NADH, P_i, morphine, naphthalene, and naphthol, according to the methods presented in Table IV, a serious experimental difficulty was encountered. The apparent solute spaces were larger than the total intramicrosomal water space. A certain decrease of the solute spaces occurred by adding unlabeled carrier, indicating adsorption. In the case of substances like naphthalene and naphthol, the low water solubility of the compounds limited the possibility of suppressing adsorption by adding increasing amounts of unlabeled substance. For the nucleotides and for P_i, the adsorption was so effective that the apparent solute space was somewhat larger than the total internal water content of the microsomes even at the very highest carrier concentrations. For this reason, the pellet content of these substances could not be related to the intramicrosomal water space in a meaningful way in the following experiments.

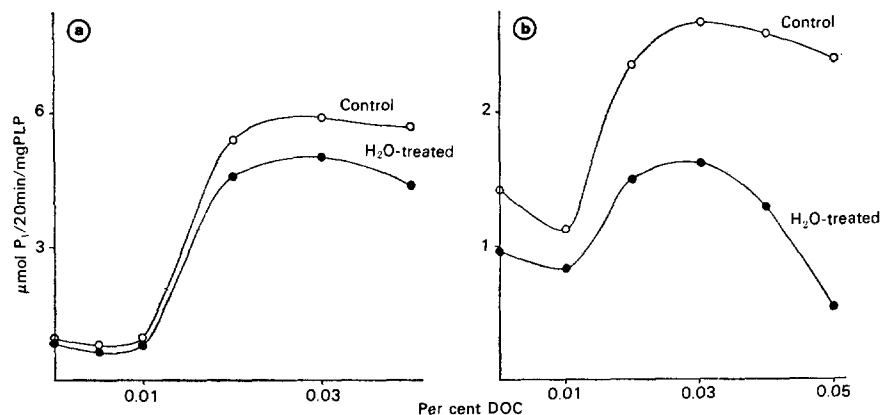During the enzymic hydrolysis of IDP to

Figure 4 Effect of DOC on the (a) IDPase and (b) G6Pase activities of H_2O-treated microsomes. The IDPase and G6Pase activities of control and H_2O-treated microsomes (both suspended in 0.25 M sucrose) were measured in the presence of increasing DOC concentrations as described previously (50).

IMP, the formation and localization of inorganic phosphate in supernate and pellet were measured upon interruption of the reaction at various intervals and after sedimenting the microsomes. In order to determine the localization of the IMP formed, labeled IDP was included in the incubation medium and the total radioactivity was measured in supernate as well as pellet. The total radioactivity, naturally, only gives the sum of the substrate and the reaction product. Since, however, about 70% of the IDP originally present is converted to IMP during the reaction, a sizable accumulation of IMP inside the lumen of the microsomes would easily be detected as an increase of radioactivity in the pellet and a corresponding decrease of the radioactivity in the supernate.

The concentration of P_i increased linearly with time in the supernate (Fig. 5). The concentration of P_i in the pellet remained practically unchanged at a low value during the entire reaction. The levels of the radioactive nucleotides remained unchanged in both supernate and pellet, and there was, thus, no evidence for any appreciable accumulation of IMP in the vesicle lumen. The relatively low values in the pellet mean that only a few percent of the total amount of substrate content in the suspension

were found in the pellet fraction. Identical amounts of radioactivity could be measured in the microsomal pellet when labeled IMP and PO_4 were simply mixed with the particle suspension before centrifugation in the cold.

Similar experiments were also performed with NADH-cytochrome c reductase, in which the amount of reduced cytochrome c increased progressively in the supernate during the reaction (Fig. 6). The levels of radioactive NADH and NAD remained constant. Because of the high activity of this enzyme, a diluted microsomal suspension was used, and consequently the amount of microsomal material which could be sedimented was too low for accurate determination of reaction products.

When studying the detoxication of morphine to normorphine, the formation of formaldehyde, the total levels of labeled NADPH + NADP, as well as the total levels of labeled morphine + normorphine were measured (Fig. 7). The results obtained were very similar to those obtained in Figs. 5 and 6, i.e., no appreciable concentration of reaction products in the pellet was observed.

All the experiments described so far in this section were repeated with a different technique in order to exclude the possible leakage of reaction products initially concentrated within the
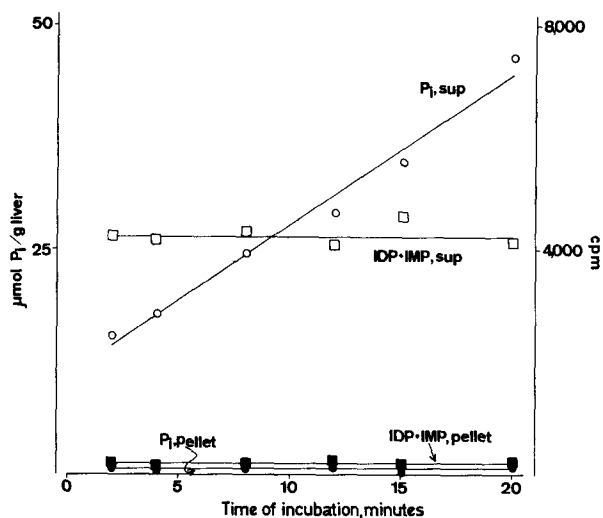

FIGURE 5 Extramicrosomal appearance of products after IDPase reaction in vitro. The incubation medium in a final volume of 4 ml contained: Tris-HCl buffer, pH 7.5, 0.1 M; IDP, 5 mM; MgCl₂, 5 mM; 0.4 ml [³H]IDP (3 μCi/ml), and enough sucrose to obtain a final concentration of 0.25 M. The medium was preincubated at 30°C and the reaction was started by adding microsomes. After incubation, the reaction was stopped by placing the tubes in an ice-water bath. After centrifugation at 105,000 g for 60 min, the supernate was decanted and the pellet suspended in 4 ml 0.25 M sucrose. Identical amounts from both pellets and supernates were taken for determination of P_i and radioactivity.
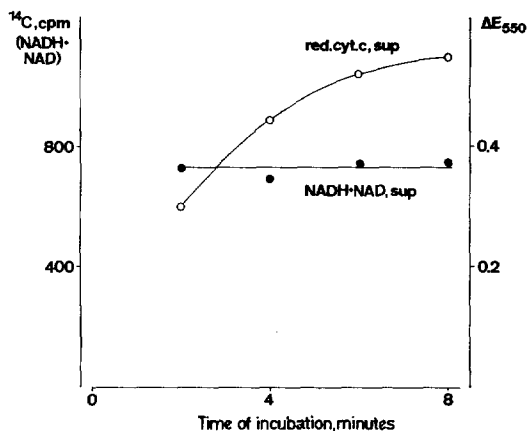
FIGURE 6 Extramicrosomal appearance of products after NADH-cytochrome c reductase reaction in vitro. The incubation medium in a final volume of 6 ml contained: potassium phosphate buffer, pH 7.5, 0.05 M; KCN, 0.33 mM; NADH, 0.1 mM; cytochrome c, 0.1 mM, and 25 µl [^{14}C]NADH (5 µCi/ml). The incubation was performed in a water bath at 30°C and the reaction was stopped by placing the tubes in an ice-water bath. 60 µl 1 M $MgCl_2$ was then added to aggregate microsomes, and centrifugation was performed at 105,000 g for 60 min. The supernate (sup) was decanted and used for the measurement of total radioactivity and for the amount of reduced cytochrome c ($red. cyt. c$). ΔE_{550} = difference in optical density at 550 nm.

microsomes resulting from membrane disruption during centrifugation. The supernate was thus separated from the microsomes by Millipore filtration at the end of the reaction. Subsequent determination of reactants in the filtrate gave results identical with those obtained from the centrifugation experiments.

In the case of naphthalene, a simple and effective method of separating the hydrophilic reaction product from the unchanged lipid soluble hydrocarbon was available (see Materials and Methods). As shown by Jerina et al. (55) the main hydroxylated product formed by microsomal detoxication of naphthalene is 1-naphthol.

A complicating factor in the study of naphthalene hydroxylation is the effective adsorption of the hydrocarbon and its hydroxylated derivative by the microsomal material. As shown in Table VII, naphthalene is totally adsorbed by the microsomes. In a reaction mixture containing approximately 10 mg of microsomal protein, about 38% of added labeled 1-naphthol was retained by the microsomes after Millipore fil-

tration of the suspension. When 1-naphthol was produced enzymatically be detoxication of naphthalene in vitro, a similar distribution of the reaction products between filtrate and microsomes was found. This is at variance with previous results obtained in in vivo experiments, where only about 5% of the reaction products were retained in pelleted microsomal material obtained from rats injected intravenously with labeled naphthalene (31). However, it would be expected that 1-naphthol formed in vivo is rapidly conjugated to the corresponding glucuronide (56). As the glucuronic acid derivative is much more hydrophilic, it would be expected that this compound has much less affinity toward the lipid-rich microsomal membranes. The polar products determined in the in vivo experiments

TABLE VII

Localization of [^{14}C]Naphthalene and Its Hydroxylated Product after Incubation with Liver Microsomes

	Percent of total counts	
	Supernate	Pellet
(1) Distribution experiment		
(a) Microsomes + naphthalene	0.7	99.3
(b) Microsomes + naphthol	62	38
(2) Detoxication in vitro		
(a) Without UDPGA	57	43
(b) With UDPGA	83	17
(3) Detoxication in vivo*	95	5

In the distribution experiments, microsomes from 1 g liver were suspended in 5 ml 0.25 M sucrose, together with 0.1 ml [^{14}C]naphthalene or [^{14}C]-naphthol. The microsomes were pelleted by ultracentrifugation or separated from the medium by Millipore filtration. The two methods gave identical results. In the experiments on in vitro detoxication, microsomes were incubated at 37°C for 15 min in the presence of an NADPH-generating system and [^{14}C]naphthalene as described previously (53). In exp. 2 b, the incubation medium contained 10 mg UDPGA in a final volume of 4 ml. The incubation was interrupted by Millipore filtration, and the radioactivity of the hydroxylated products was determined both in the total incubation medium before filtration and in the filtrate. The counts in the pellet were calculated from the difference.
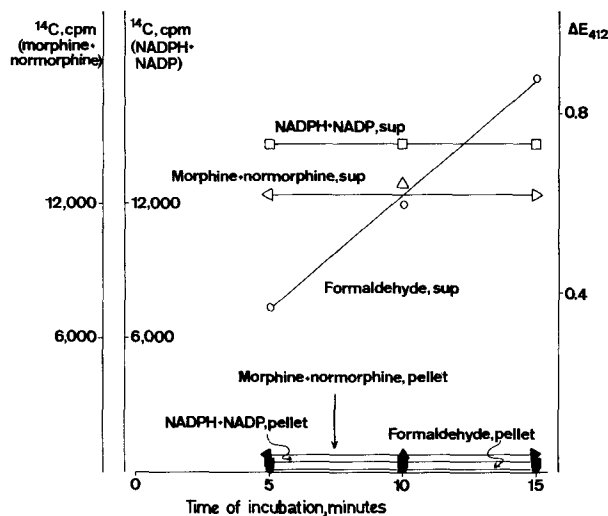
* Data taken from reference 31.

FIGURE 7 Extramicrosomal appearance of products after morphine hydroxylation in vitro. In all experimental series, two tubes (A and B) were incubated for every time point because of the two ^{14}C-labeled compounds employed. Incubation medium A in a final volume of 3 ml contained: Tris-HCl buffer, pH 7.5, 0.1 M; MgCl$_2$, 5 mM; NADPH, 1 mM; morphine-HCl, 6 mM, and 0.1 ml [^{14}C]morphine-HCl (1 μCi/ml). Incubation medium B in a final volume of 8 ml contained: Tris-HCl buffer, pH 7.5, 0.05 M; MgCl$_2$, 5 mM; NADPH, 1 mM; morphine-HCl, 6 mM, and 25 μl [^{14}C]NADPH (5 μCi/ml). Both A and B were started by adding microsomes and incubated at 30°C. Incubation was stopped by cooling. After the addition of 30 μl 1 M MgCl$_2$, centrifugation was performed at 105,000 g for 60 min. The supernate (sup) was decanted and the pellet suspended in 1.5 ml 0.25 M sucrose. Radioactivity and liberated formaldehyde were measured in the supernates and pellets of both A and B. ΔE_{412} = difference in optical density at 412 nm.

may, thus, mainly consist of the glucuronide, since this form is less efficiently adsorbed by the microsomes. Strong support for this interpretation is given by exp. 2 b of Table VII, in which UDPGA had been added to the incubation mixture during hydroxylation in vitro. Although the conditions for conjugation were probably not such as to ensure complete transformation of the 1-naphthol into the glucuronide, the content of reaction product present in the microsomes was drastically reduced. In view of the effect of UDPGA and the results of the in vivo experiments, there seems no reason to believe that the products of naphthalene hydroxylation are concentrated inside the microsomal vesicles. It should be pointed out, however, that in the absence of UDPGA a major part of the reaction products are released into the supernate.

DISCUSSION

This paper describes the permeability properties of the microsomal membranes toward various substances, the nature of the sucrose space, as well as the localization of metabolic products found after certain enzymic reactions in vitro.

Several lines of approach have demonstrated that the membranes of certain subcellular particles like nuclei (57), the outer membrane of mitochondria (58, 59), and peroxisomes (60) are freely penetrable to uncharged molecules such as sucrose. On the other hand, the inner mitochondrial membranes (61, 62) and parts of the lysosomal structures appear to be inaccessible to sucrose (44). The situation for microsomes is less simple. Using labeled sucrose, Share and Hansrote (63) concluded that these structures lack osmotic response and that approximately 80% of the total water space in 0.3 M sucrose is permeable to this substance. Maude found that 16–27% of the renal microsomal pellet water was inaccessible to sucrose and that only a small fraction of the total water (0.3–4.2%) was involved in osmotic shifts (64). Evidence for the osmotic behavior of microsomes from liver (3) has, on the other hand, been provided by photometric methods as well as by electron microscope

studies (1). Johnson and Tedeschi (65), in a more recent photometric investigation, found that six-carbon nonelectrolytes rapidly (>1 s) penetrated membranes of liver microsomes, while sucrose and raffinose exhibited a slow penetration. These authors concluded that photometric measurements could not be applied to kinetic studies of liver microsomal penetrability in a simple manner. Further, the transmission of incident or scattered light by such a suspension will be greatly influenced by such factors as aggregation or loss of high molecular weight substances. Similarly, Gamble and Garlid (66) demonstrated that while the inner mitochondrial membrane, although having a different composition than microsomal membranes, was practically inaccessible to sucrose when exposed for less than 5 min, sucrose gains entrance into the inner compartment on longer incubation.

Our studies confirm the findings of Share and Hansrote insofar as the sucrose space make up 77% of the total intramicrosomal water, leaving almost no osmotic space when correction is made for hydration of the matrix. The period of incubation is clearly decisive when performing studies of this kind. The demonstration of a large sucrose-accessible space by the use of a labeled compound need not conflict with the spectrophotometric evidence when considering that equilibration occurs in the former experiments during 2 h centrifugation. In a study of osmotic flow across permeable cellulose membranes, Durbin (67) found the rate of diffusion of water to be much faster than that of sucrose. The great differences in the diffusion rate of water and sucrose may cause an initial swelling of the microsomal vesicles before equilibration occurs (36).

The possibility that the sucrose-nonaccessible space represents intact vesicles and the sucrose-accessible space mechanically disrupted vesicles in a heterogeneous population may be excluded, since this would hardly account for the exclusion of acetate and mevalonate by the entire vesicle population. Finally, it seems highly improbable that the sucrose-accessible space can be attributed to an unspecific adsorption. The sucrose occluded in the microsomal pellet is easily removed by dialysis. Further, increasing the amount of unlabeled carrier to high concentration does not change the results. If sucrose did not penetrate the vesicles, an increase in the concentration

would be expected to cause osmotic shrinkage, which is not the case. Because of the presence of an initial osmotic effect followed by a slower, gradual penetration of sucrose into the microsomes, the behavior of these subcellular particles upon gradient centrifugation would be expected to be extremely complex. On the other hand, as expected, nonpenetrating, charged substances do induce an osmotic response of the vesicles.

Certain methodological problems should be considered when performing permeability studies with the ultracentrifugation technique described.

(a) When studying the distribution of various labeled compounds other than sucrose, it is of the utmost importance to exclude artifacts such as unspecific adsorption. In fact, adsorption effects severely limit the number of substances that can be studied by this method.

(b) Unfortunately, the sedimentation of microsomes requires such a long centrifugation time that it is not possible to study the kinetics of penetration during the initial phase by investigating the distribution of a labeled solute.

(c) The accuracy of the results is greatly influenced by the amount of microsomal material used, and subfractions of rough and smooth microsomes separated by zone centrifugation (40, 68) contain too little material for such measurements.

Evidently, microsomal membranes can be penetrated by uncharged molecules of a molecular weight of at least 600, whereas larger polymers like inulin and dextran, with a molecular weight of above 5,000, could not pass the membrane barrier. The charged molecules of acetate and mevalonate were completely excluded from the intramicrosomal water space. The situation for smaller electrolytes has not been clarified because of adsorption artifacts.

Of great interest was the finding that the procedure of Schramm et al. (41) could render the microsomal membranes permeable to acetate and mevalonate and probably also to G6P. Since this treatment must be considered as a relatively mild one, i.e. not causing denaturation or inactivation of enzyme proteins, it is possible that similar changes of permeability may also occur in vivo in a number of pathological conditions. Changes in the morphological appearance of ER have been described in detail in a number of in vivo experimental systems, e.g., after feeding with low protein diet (69) and after administra-

tion of carbon tetrachloride (70), dimethyl-nitrosamine (71), and thioacetamide (72).

The results with IDPase and G6Pase show that while there is a change in the permeability of the membrane, the DOC activation effect remains unchanged after H₂O treatment. Clearly, the free penetration of substrate into the microsomal lumen does not mean that the substrate has free access to the enzyme.

The absence of passive penetration across the membrane does not necessarily preclude an active translocation of charged substances to the inner side of the microsomal vesicles. Recently, Behrens and Leloir (73) and Richards et al. (74) identified a polyprene intermediate, dolichol monophosphate, from liver which seems to be involved in the translocation of activated sugars across membranes. This carrier is present in all the different types of microsomal membranes and also in other subcellular organelles (75).

In our in vitro experiments, we found no evidence for an appreciable accumulation of the products in the lumen from the reactions catalyzed by IDPase, NADH-cytochrome c reductase, and NADPH-dependent hydroxylation of morphine. The concentration of labeled products present in the pelleted material only amounted to a small fraction of the total after most of the substrate had been converted. Theoretically, it is possible that both substrates and products freely penetrate the membrane, causing a rapid equilibration. Unless an active transport is involved, this interpretation seems less probable in view of the fact that the membrane is impermeable even to small charged organic molecules like acetate and mevalonate. On the other hand, rapid equilibration may conceivably occur with small inorganic anions like phosphate.

It seems established, however, that the release of various substances into the supernatant is not due to secondary damage caused by the preparation procedures, as demonstrated by the results of the Millipore filtration experiments and the impermeability of the pelleted microsomes to acetate.

A strongly nonpolar hydrocarbon like naphthalene is rapidly dissolved in the microsomal membrane as demonstrated by mixing an aqueous solution of naphthalene with microsomes. Also, naphthol is readily adsorbed, although less efficiently than the parent hydrocarbon (Table VII). The concentration in the pellet of hydroxyl-

ated product formed in vivo is much lower than in the corresponding in vitro test. Conjugation with glucuronic acid to a more hydrophilic product which is less readily adsorbed by the microsomal material offers a plausible explanation of this effect. The major pathway for release of the products seems to be into the cytoplasm.

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