Lipid composition and turnover of rough and smooth microsomal membranes in rat liver

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ABSTRACT Subfractions of rat liver microsomes (rough, smooth I, and smooth II), isolated in a cation-containing sucrose gradient system, were analyzed. After removal of adsorbed and luminal protein, these subfractions had the same phospholipid/protein ratio, about 0.40. Both the classes and the relative amounts of phospholipids were similar in the three subfractions, but the relative amounts of neutral lipids (predominantly free cholesterol and triglycerides) were higher in smooth I and especially in smooth II than in rough microsomes. Various pieces of evidence indicate that the neutral lipids are tightly bound to the membranes.

Glycerol-^{3}H was incorporated into the phospholipids of the rough and smooth I microsomes significantly faster than into those of the smooth II membranes; ^{32}P incorporation followed a similar but less pronounced pattern. Acetate-^{3}H was incorporated into the free cholesterol of smooth I microsomes only half as fast as into the other two subfractions. Injection of phenobarbital increased the cellular phospholipid *and* neutral lipid content in the rough and smooth I, but not in the smooth II microsomes. Consequently, the neutral lipid/phospholipid ratio of all three subfractions remained unchanged after phenobarbital treatment.

It is concluded that the membranes of the rough and the two smooth microsomal subfractions from rat liver have a similar phospholipid composition, but are dissimilar in their neutral lipid content and in the incorporation rate of precursors into membrane lipids.

SUPPLEMENTARY KEY WORDS neutral lipid · membrane components · smooth I and II · phenobarbital

THE LIPIDS of the microsomal fraction of rat liver constitute about 30% of its dry weight and consist predominantly of phospholipids (1). The composition and turnover of microsomal PL have been studied thoroughly, but the neutral lipid composition has been investigated only from certain specific points of view.

During recent years, it has become clear that the microsomal fraction is heterogeneous. The two main constituents of the fraction are the rough- and smooth-surfaced vesicles, of which the former have ribosomes attached to the outer surface (2). The rough vesicles undoubtedly derive from the rough-surfaced endoplasmic reticulum (ER), while the smooth vesicles originate mainly from the smooth-surfaced ER, at least in liver. The total microsomal fraction can be separated in a monovalent cation-containing discontinuous sucrose gradient into rough and smooth microsomal subfractions, and the latter can be further separated in a divalent cation-containing gradient into smooth I and smooth II subfractions (3). Enzymically, rough and smooth I microsomes are similar from many points of view, although there may be differences with regard to certain metabolic reactions such as glycoprotein synthesis and hydroxylations (3–5). On the other hand, smooth I and smooth II microsomes exhibit pronounced differences in the enzymic pattern, e.g. for certain phosphatases and electron transport enzymes. During the biogenesis of intracellular membranes, the newly synthesized proteins appear preferentially in the rough microsomal fraction, after which they seem to be transferred to the smooth microsomes (6–8). This sequence of events includes both total membrane proteins and certain individual constitutive enzymes. Various microsomal components also play an important role in the transport of newly synthesized serum proteins. After synthesis of the albumin by the bound ribosomes, the protein is transferred to the channels of the rough ER, followed by a further transport to the smooth ER (9). Both smooth I and smooth II microsomes participate in the albumin transport, for the labeled albumin from the rough micro-

Abbreviations: Ch, free (unesterified) cholesterol; CE, cholesteryl esters; DOC, deoxycholate; ER, endoplasmic reticulum; FFA, free fatty acids; PL, phospholipid(s); TG, triglycerides; NL, neutral lipid(s).

somes is transferred simultaneously into both smooth subfractions (10).

Lipids are known to play an indispensable part in keeping the microsomal membrane structure intact (11) and in influencing the kinetic and thermodynamic properties of many constitutive enzymes (7, 12–16). Since it appeared that the lipid composition and turnover might display specific patterns in various membranes, the lipids of microsomal subfractions were analyzed. A preliminary report of this work has appeared (17).

MATERIALS AND METHODS

Isolation of Microsomes and Microsomal Subfractions

Microsomes from livers of rats fasted for about 20 hr were prepared as described previously (18). When phenobarbital-treated rats were used, 8 mg of phenobarbital/100 g body weight was injected intraperitoneally daily in a single dose.

The microsomes were subfractionated as described earlier, with certain modifications (3, 19). A 25% homogenate of liver in 0.25 M sucrose was centrifuged at 10,000 *g* for 20 min. Sufficient 1 M CsCl was added to the supernate to give a final concentration of 15 mM. 8 ml of this supernate was layered over 3.5 ml of 1.30 M sucrose–0.015 M CsCl. After centrifugation at 250,000 *g* for 60 min (Christ Omega I ultracentrifuge, rotor 60, tube angle 34°), the reddish, clear upper phase was removed with a pipette provided with a rubber aspirator and discarded. The entire fluffy double layer at the gradient boundary (about 1 ml) was collected together with about 0.5 ml of the 1.30 M clear sucrose solution. When indicated, this suspension was diluted with 0.25 M sucrose and centrifuged at 105,000 *g* for 90 min to give a pellet; resuspended, this material was designated *total smooth microsomes*.

When further subfractionation was performed, the fluffy layers from two centrifuge tubes were combined, diluted with distilled water to 7 ml, mixed with 1 M $MgCl_2$ to give a final concentration of 7 mM, and layered over 4.5 ml of 1.15 M sucrose–0.007 M $MgCl_2$. After centrifugation at 250,000 *g* for 30 min, the clear upper phase was removed and discarded. The thin fluffy layer at the gradient boundary was collected, diluted with 0.25 M sucrose, and recentrifuged at 105,000 *g* for 120 min. After resuspension of the pellet in 0.25 M sucrose, we obtained the fraction called *smooth II microsomes*.

The 1.15 M sucrose of the gradient containing Mg^{2+} was decanted, and, after resuspension of the pellet, the fraction was called *smooth I microsomes*.

Since the pellet obtained in the first gradient centrifugation in the presence of Cs^+ sometimes had a loose surface, the 1.30 M sucrose was not removed completely: a few drops of the sucrose were left behind. Distilled water was added to bring the sucrose concentration to 0.25 M, and the pellet, called *rough microsomes*, was resuspended.

The purity of the subfractions was checked by electron microscopy and by chemical and enzymic analysis. The results were in agreement with previous findings (3).

To separate membrane components from ribosomes and vesicular content, we treated both rough and total smooth microsomes (about 4 mg of protein per ml) with 0.26% deoxycholate (DOC) by a modification of the procedure of Ernster, Siekevitz, and Palade (18), since CsCl decreases the effect of DOC on rough microsomes (8). The treated suspensions were centrifuged for 4 hr at 105,000 *g* to yield tightly packed pellets of ribosomes and membranes, and supernates consisting of nonmembranous adsorbed protein, microsomal contents, and the solubilized part of the membranes. The pellets were rinsed three times with 0.25 M sucrose and used for lipid extraction.

Washing Procedure

In order to remove adsorbed protein, mainly hemoglobin, we resuspended the microsomal pellets in 0.15 M Tris buffer, pH 8.0 (7). The suspension was then centrifuged at 60,000 *g* for 2 hr and the pellet was resuspended in distilled water; this removed the contents of the vesicles, mainly albumin (20). Total microsomes from 1 g of liver were made up in 40 ml, which corresponds to about 0.5 mg of protein per ml. The subfractions were diluted to give the same final protein concentration. After incubation at 30°C for 15 min, the suspensions were cooled (21) and centrifuged at 60,000 *g* for 2 hr. The 15 min incubation in the hypotonic medium assured the maximal swelling of vesicles, making them easily accessible to cold-induced rupture.

Phospholipids

The pellets from the different fractions were extracted with chloroform–methanol 2:1 in the presence of 0.05% butylated hydroxytoluene as antioxidant and in a nitrogen atmosphere. The washing procedure of Folch, Lees, and Sloane Stanley was employed (22).

The phospholipids (PL) were separated on paper impregnated with silicic acid according to Marinetti (23, 24). The solvent system consisted of diisobutylketone–acetic acid–0.9% NaCl 40:25:5. 0.05% butylated hydroxytoluene was also present as an antioxidant. The PL were detected both by radioautography, since the lipids were always labeled with ^{32}P, and by staining with Rhodamine 6G.

The PL were eluted from the chromatograms with methanol–HCl (25, 26), and the amounts of phosphorus were measured according to Marinetti (23). The PL were

characterized by two-dimensional chromatography of the eluted samples, after deacylation, according to Dawson (27).

PL were measured by determination of the phosphorus in the washed chloroform phase and the value was multiplied by 25.

In experiments with ^{32}P, a solution of carrier-free isotonic sodium phosphate-^{32}P (Radiochemical Centre, Amersham, England) was injected intraperitoneally (0.5 mc/100 g body weight). The livers were removed after 1 hr and fractionated. Incorporation into all individual phospholipids was linear during this time. Aliquots of the methanol–HCl eluate were plated and counted in a Geiger–Müller gas-flow counter. Radioactivity was corrected for decay.

Glycerol-2-^{3}H (50 mc/mmole) from the Radiochemical Centre was diluted with sterile Ringer's solution. After intraperitoneal injections (20 μc/100 g at indicated times) the rats were decapitated. The liver was subfractionated and the lipids were extracted in the manner described above. The PL were separated from the neutral lipids (NL) by silicic acid column chromatography of the washed chloroform phase (28). The methanol eluate was used for measurement of lipid P and of radioactivity in a toluene scintillation mixture (29).

Neutral Lipids

After separation of neutral lipids from PL, aliquots of chloroform were evaporated in a nitrogen atmosphere and redissolved in chloroform. They were fractionated on silica gel-loaded paper (H. Reeve Angel and Co., Clifton, N. J.) as described by Marinetti (30), with *n*-heptane–diisobutylketone–acetic acid 85:15:1. 0.05% butylated hydroxytoluene was also added. Spots were detected by staining with Rhodamine 6G. Chromatographically pure standards were applied simultaneously for identification.

Free cholesterol (Ch) and cholesteryl esters (CE) were extracted with $FeSO_4$-saturated acetic acid, and the amount of cholesterol was determined (31). Triglycerides (TG) were measured after alkaline hydrolysis (32); tripalmitate (Sigma) was used as a standard. Free fatty acids (FFA) were titrated as described by Trout, Ester, and Friedberg (33). The standard was purified palmitic acid (kindly supplied by Dr. L. Carlsson, Stockholm). The NL were measured both after methanol extraction from the paper (60°C, 60 min) and in the original chloroform with identical results.

Sodium acetate-^{3}H (650 mc/mmole) from the Radiochemical Centre was injected intraperitoneally (1 mc/100 g) and the livers were removed at the times indicated. The subfractionations and separation of Ch were performed as described above.

Protein and RNA

Protein was determined (34) with bovine serum albumin as standard. Ribonucleic acid (RNA) was extracted and measured as described previously (6).

RESULTS

Phospholipid Content

The majority of total microsomal PL, about 55%, are recovered in the rough, 35% in the smooth I, and 5–8% in the smooth II subfractions (Table 1). Since a part of the protein in the rough microsomes derives from the attached ribosomes, the PL/protein ratio is lower in the rough subfraction than in the two smooth counterparts. A sizable part of the "microsomal" protein is not a true part of the membrane—it may be adsorbed (35), contained in the lumen (36), or be part of the attached ribosomes—and for this reason the subfractions were subjected to a washing procedure. This results in about 45% loss of protein, a 90% recovery of PL, and a 90% loss of RNA. The removal of the nonmembranous protein results in a similar PL/protein ratio, about 0.4, for all three types of microsomal membranes. Since the washing procedure removed 90% of the microsomal RNA, correction of the values for remaining ribosomal protein does not affect this conclusion.

TABLE 1 TOTAL PROTEIN, RNA, AND PHOSPHOLIPID CONTENT OF MICROSOMAL SUBFRACTIONS

	Before Washing					After Washing				
Fraction	Protein	PL	RNA	PL/Protein	RNA/Protein	Protein	PL	RNA	PL/Protein	RNA/Protein
	mg/g liver					*mg/g liver*				
Total	18.6	5.10	4.11	0.27	0.22	10.0	4.34	0.60	0.43	0.06
Rough	10.9	2.84	3.31	0.26	0.30	5.85	2.34	0.35	0.40	0.06
Smooth I	5.92	1.89	0.49	0.32	0.08	3.92	1.61	0.20	0.41	0.05
Smooth II	0.77	0.27	0.05	0.35	0.06	0.37	0.16	0.02	0.43	0.04

For washing, the different subfractions were resuspended in 0.15 M Tris buffer, pH 8.0, and centrifuged at 60,000 *g* for 120 min. The pellets were resuspended in distilled water (about 20 mg of protein/40 ml) and incubated at 30°C for 15 min. After cooling and centrifugation at 60,000 *g* for 120 min, the pellets were analyzed for protein, PL, and RNA.

TABLE 2 PHOSPHOLIPID COMPOSITION OF MICROSOMAL SUBFRACTIONS

	Total		Rough		Smooth I		Smooth II	
	Lipid P	% of Total	Lipid P	% of Total	Lipid P	% of Total	Lipid P	% of Total
	μg/g liver		*μg/g liver*		*μg/g liver*		*μg/g liver*	
Total	255 ± 18.1	100	111 ± 14.2	100	77.0 ± 5.5	100	19.8 ± 2.8	100
PE	42.7 ± 1.8	19.0	21.2 ± 1.5	19.3	13.5 ± 1.0	17.5	3.6 ± 0.44	18.0
PS	19.6 ± 2.0	8.5	7.8 ± 1.2	7.1	6.3 ± 0.96	8.0	1.5 ± 0.21	7.5
PC	108.1 ± 8.5	47.5	52.0 ± 5.5	47.0	36.4 ± 3.5	46.4	9.1 ± 1.1	45.5
Sph	12.1 ± 0.84	5.8	6.0 ± 0.71	5.5	5.1 ± 0.62	6.6	2.3 ± 0.32	11.1
PI	22.5 ± 2.5	10.0	11.3 ± 1.3	10.1	8.5 ± 0.68	11.1	1.6 ± 0.22	7.6
Recovery		90.8		89.0		89.6		89.7

The values are the means ± SEM (n = 8). PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PC, phosphatidyl choline; Sph, sphingomyelin; PI, phosphatidyl inositol.

TABLE 3 INCORPORATION OF ^{32}P INTO INDIVIDUAL PHOSPHOLIPIDS OF MICROSOMAL SUBFRACTIONS

	Total		Rough		Smooth I		Smooth II	
	SA*	% of Total Radioactivity	SA*	% of Total Radioactivity	SA*	% of Total Radioactivity	SA*	% of Total Radioactivity
Total	3500 ± 240	100	3850 ± 550	100	3660 ± 250	100	3280 ± 390	100
PE	5210 ± 240	24.9	5200 ± 342	23.9	4500 ± 310	20.6	4300 ± 229	22.2
PS	2820 ± 283	5.8	3580 ± 200	5.6	3080 ± 300	5.6	3070 ± 209	4.8
PC	4360 ± 334	53.5	4550 ± 510	52.0	4070 ± 265	53.0	4010 ± 251	52.0
Sph	2410 ± 210	5.7	1910 ± 130	5.1	1715 ± 186	6.4	1190 ± 50	7.0
PI	860 ± 50	3.9	865 + 55	4.1	810 ± 53	4.6	845 ± 29	3.5
Recovery		93.8		90.7		90.2		89.5

The rats received 0.5 mc of carrier-free ^{32}P per 100 g body weight, intraperitoneally. They were decapitated after 60 min. The values shown are means ± SEM (n = 8).

* Specific activity in cpm/μg of lipid-P.

Phospholipid Composition and Incorporation of ^{32}P and Glycerol-^{3}H

The two main phospholipids of total microsomes are phosphatidyl choline and phosphatidyl ethanolamine; the other components are phosphatidyl serine, phosphatidyl inositol, and sphingomyelin (Table 2). Moreover, a small amount of phosphatidic acid, about 1% of the total PL, is detected in the chromatographic separation (not shown in table). All the five main phospholipids are present in the various microsomal membranes. As regards the quantitative distribution, no differences between the subfractions are apparent. The only possible exception is sphingomyelin, of which there is proportionately somewhat more in the smooth II microsomes.

In vivo incorporation of ^{32}P into phospholipids is shown in Table 3. No striking difference is apparent in the rate of incorporation into total PL among the subfractions, but the smooth II microsomes exhibit a 10–15% lower rate of labeling. Minor differences are found on comparison of the individual phospholipids. The phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, and sphingomyelin of the two smooth subfractions display a somewhat lower rate of incorporation in relation to the rough microsomes. However, these differences are moderate and without statistical significance. The high incorporation of ^{32}P into the phosphatidyl choline and phosphatidyl ethanolamine of liver microsomes has been described previously (6). As Table 3 demonstrates, this is valid not only for the total microsomes but also for the rough and both smooth subfractions. The percentage recovery of radioactivity is similar in the various phospholipids, regardless of the type of subfraction.

When glycerol-^{3}H is injected intraperitoneally, the rate of incorporation into the subfractions displays a different pattern (Fig. 1). The total PL fraction, separated from NL by silicic acid column chromatography, exhibits a similar rate of labeling in the rough and the smooth I fractions, but the rate of incorporation into smooth II microsomes is significantly (about 50%) lower.

Total liver extract is known to contain a small amount of glycolipid, predominantly lactosylceramide (37). If the microsomal membrane contains glycolipids, they would be included in the PL fraction after silicic acid chromatography. In this case, the possible incorporation of glycerol-^{3}H into glycolipids would influence the results

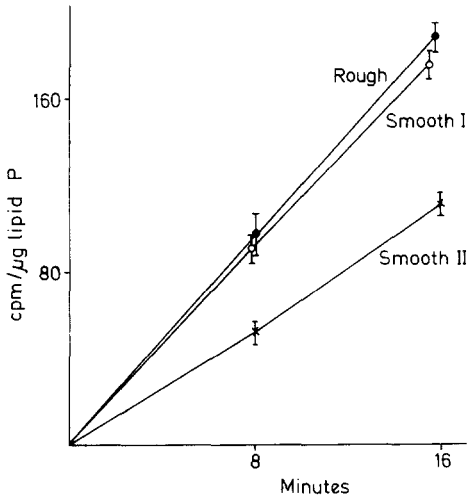

FIG. 1. Incorporation of glycerol-^{3}H into PL of microsomal subfractions. The rats received glycerol-2-^{3}H, 20 μc/100 g body weight, intraperitoneally. The values are taken from four experiments; the vertical bars represent SEM.

described in Fig. 1. In order to investigate this possibility we separated glycolipids from the PL fraction by the method of Vance and Sweeley (38). Glycerol-^{3}H incorporation into the glycolipid-free PL fraction gave results identical with those in Fig. 1, thereby excluding significant error due to glycolipid incorporation.

Neutral Lipid Composition

NL were fractionated on silica gel-loaded paper, a method described recently by Marinetti (30). Microsomes contain Ch, CE, TG, and FFA. Occasionally, a diffuse spot appears just below the Ch, which, to judge by the R_f value, probably consists of diglycerides. The two major components are Ch, 0.6 mg/g, and TG, 0.5 mg/g (Table 4). The amount of CE is only 10% of the total cholesterol both in the total microsomes and the subfractions. A small amount of FFA is present, but this may represent a hydrolysis product resulting from the isolation procedure. The sum of the amounts of Ch in the two smooth microsomal subfractions exceeds the amount present in the rough microsomes; the PL content exhibits a reverse distribution. The Ch/PL ratio will therefore be higher in the two smooth subfractions, as is the TG/PL ratio (Table 4).

The finding that the various microsomal subfractions exhibit a different NL content is of importance only if NL represent a true membrane component, not a contaminant from the cytoplasm. Further analysis of the cytoplasmic NL distribution therefore appeared to be of interest.

The microsomal supernate, recentrifuged at 105,000 *g* for 60 min, contains a sizable amount of NL, especially TG, but very little PL (about 0.06 mg/g liver). The presence of the latter can be attributed to slowly sedimenting membrane fragments which are part of the postmicrosomal fractions (2). The supernate from liver homogenate of rats starved for 20 hr contains 0.13–0.16 mg of total cholesterol and 1.1–1.5 mg of TG per g of liver. To investigate the possibility of cytoplasmic contamination of microsomes, we employed the washing procedures generally used to remove the nonmembranous material, as well as treatment with a low concentration of DOC (Table 5). None of the washing procedures decreased the total PL, TG, or total cholesterol content. 0.26% DOC is known to solubilize a part of the microsomal membrane, including some enzymes and PL (18). Table 5 shows that 30–40% of each lipid class was recovered in the soluble fraction, which again indicates that these lipids in the microsomal fractions are not the result of an adsorption from the soluble cytoplasm.

In order to exclude the possibility that 0.26% DOC solubilizes certain microsomal membranes but not others, the treatment was performed on rough as well as smooth microsomal subfractions. Both types of membrane were affected similarly (40–45% solubilization of the PL).

Incorporation of Acetate-^{3}H and Glycerol-^{3}H into Neutral Lipids

The incorporation of acetate-^{3}H into Ch was studied after isolation of the microsomal subfractions (Fig. 2). In contrast to the results for glycerol-^{3}H incorporation into the total PL, the smooth I microsomes incorporated

TABLE 4 NEUTRAL LIPID COMPOSITION OF MICROSOMAL SUBFRACTIONS

	PL	Ch	CE	TG	FFA	Ch:PL	TG:PL
			mg/g liver				
Total	6.3 ± 0.6	0.570 ± 0.048	0.071	0.462 ± 0.046	0.071	0.09	0.07
Rough	3.2 ± 0.2	0.224 ± 0.035	0.032	0.200 ± 0.018	0.032	0.07	0.06
Smooth I	2.2 ± 0.2	0.241 ± 0.039	0.022	0.198 ± 0.018	0.033	0.11	0.09
Smooth II	0.38 ± 0.10	0.064 ± 0.010	0.011	0.042 ± 0.004	0.007	0.17	0.11
Recovery	92.0%	39.0%	91.5%	94.9%	101.4%		

Values are means ± SEM (n = 7).

TABLE 5 EFFECT OF VARIOUS TREATMENTS ON LIPIDS OF TOTAL MICROSOMES AND MICROSOMAL SUBFRACTIONS

Expt.	Fraction	Treatment	PL	TG	Total Cholesterol
			mg/g liver		
1.	Total	None	6.1	0.47	0.61
	"	Tris buffer, 0.15 M, pH 8.0	6.3	0.39	0.60
	"	H_2O, 30°C, 15 min	6.1	0.47	0.66
	"	KCl, 0.15 M	6.3	0.42	0.59
	"	Sucrose, 0.25 M	6.0	0.45	0.61
2.	Total	None	6.2	0.41	0.63
	"	0.26% DOC			
		Soluble	2.3	0.13	0.27
		Insoluble	3.9	0.28	0.36
	Rough	None	3.2		
	"	0.26% DOC			
		Soluble	1.5		
		Insoluble	1.7		
	Smooth	None	3.0		
	"	0.26% DOC			
		Soluble	1.2		
		Insoluble	1.8		

Total microsomes were washed by resuspending the pellets in the various media (Tris buffer, KCl, and sucrose) by slight motor-driven homogenization in a volume of 10 ml/g of liver and recentrifugation at 105,000 *g* for 90 min. For treatment with water the pellet was resuspended in distilled water (40 ml/g liver), incubated at 30°C for 15 min, cooled in ice, and recentrifuged at 105,000 *g* for 90 min. DOC treatment: the total microsomes or microsomal subfractions were centrifuged at 105,000 *g* for 4 hr in the presence of 0.26% DOC in 0.25 M sucrose (about 4 mg protein/ml).

less acetate into Ch than the rough or smooth II microsomes.

Studies of incorporation of glycerol-^{3}H into TG (Fig. 3) presented further evidence that the TG are tightly bound to the membranes, so that it is unlikely that adsorption can explain their presence in microsomes. When radioactivity is measured for a period of 60 min after injection of glycerol-^{3}H, microsomal and supernatant TG exhibit very different patterns of labeling (Fig. 3). The microsomal TG displays a rapid rate of incorporation with a peak after 16 min followed by a rapid decrease. The incorporation rate of supernatant TG is considerably lower, and the degree of labeling reaches a peak after about 30 min. Up to 15 min after injection, the specific activity in the supernate is only one-fourth that of the microsomal TG. The two fractions do not exhibit a similar specific activity until about 60 min have elapsed. This pattern of labeling indicates that the isolated microsomes cannot be contaminated with cytoplasmic TG to any great extent. Fig. 3 also shows the remarkable difference in rate of glycerol-^{3}H labeling of microsomal PL and TG, the latter being considerably higher.

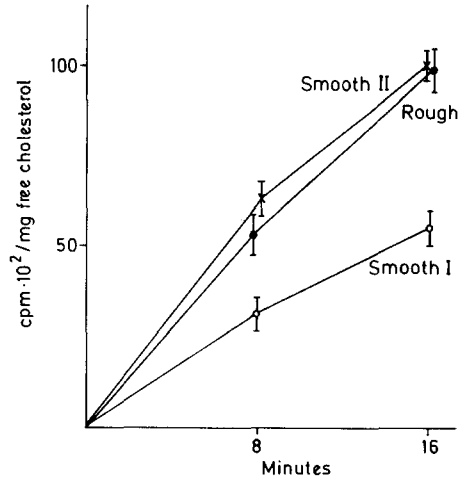

FIG. 2. Incorporation of acetate-^{3}H into Ch of microsomal subfractions. The rats received acetate-^{3}H, 1 mc/100 g, intraperitoneally. The values are taken from three experiments; vertical bars represent SEM.

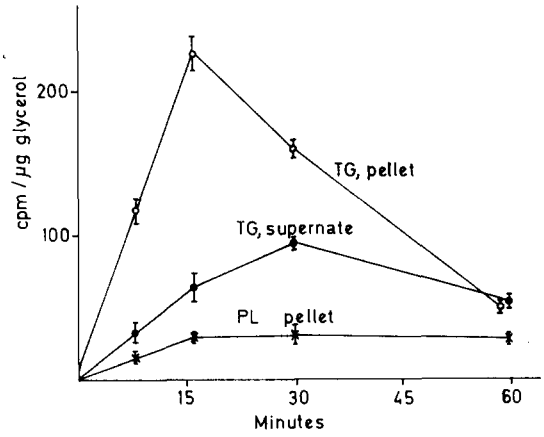

FIG. 3. Incorporation of glycerol-2-^{3}H into TG of the microsomal pellet and supernate, and into PL of microsomes. The rats received glycerol-2-^{3}H, 20μc/100g, intraperitoneally. The values are taken from three experiments; vertical bars represent SEM.

Phenobarbital Treatment

Repeated treatment of rats with phenobarbital doubles the amounts of protein and PL in the liver microsomal fraction, with a concomitant increase in the number of ER membranes seen in the electron micrographs (39). In order to determine whether any changes in lipid composition occur under these conditions, we analyzed the lipids of the microsomal subfractions. The PL concentration per g of liver increases both for the rough and smooth I fractions, but not for the smooth II microsomes (Fig. 4). Simultaneous protein measurements (not shown) gave the same pattern, with the result that the PL/protein ratio was constant at all time points.

Five injections of phenobarbital also increased the amount of TG and total cholesterol in the rough and smooth I membranes; again, there was no increase in

TABLE 6 NEUTRAL LIPIDS OF MICROSOMAL SUBFRACTIONS AFTER PHENOBARBITAL TREATMENT

Fraction	Control				Phenobarbital			
	Total Cholesterol		Triglycerides		Total Cholesterol		Triglycerides	
	mg/liver	*mg/mg PL*	*mg/g liver*	*mg/mg PL*	*mg/g liver*	*mg/mg PL*	*mg/g liver*	*mg/mg PL*
Total	0.630	0.090	0.492	0.071	0.950	0.083	0.763	0.067
Rough	0.228	0.067	0.194	0.060	0.370	0.062	0.325	0.056
Smooth I	0.225	0.103	0.195	0.087	0.410	0.110	0.317	0.085
Smooth II	0.070	0.171	0.046	0.111	0.065	0.151	0.046	0.107

Phenobarbital was injected intraperitoneally daily for 5 days (8 mg/100 g). Values are the averages of two experiments.

the smooth II fraction (Table 6). Consequently, the NL/PL ratios remain unchanged.

DISCUSSION

Although the microsomal subfractions isolated and analyzed in this study are similar to total microsomes in several respects, they also exhibit some definite differences. Thus, the total microsomes from rat liver are shown to consist of separate entities characterized not only by sedimentational, ultrastructural, and enzymic properties but also by lipid composition and turnover. The exact cellular origin of the liver microsomal subfractions is not fully established. After homogenization, a number of cellular membranes, such as smooth-surfaced ER, Golgi, and plasma membranes, are seen as smooth vesicles of similar morphological appearance. The preponderance of Golgi structures in the smooth microsomes of pancreas (40) and epididymis (41), as well as of plasma membranes in the smooth microsomal fraction of kidney cortex (42), has been described. Obtaining the mitochondrial supernate from liver by centrifuging a 25% (w/v) homogenate in 0.25 M sucrose at 2×10^5 *g*-min avoids a significant contamination of microsomal subfractions by plasma membranes, outer and inner mitochondrial membranes, or lysosomes (10).

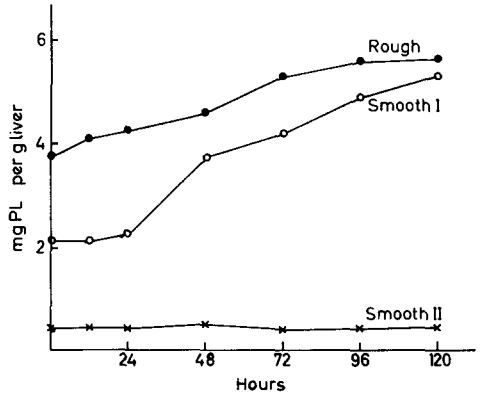

FIG. 4. Distribution of PL in microsomal subfractions after phenobarbital treatment. The rats were injected intraperitoneally once daily at zero time and every subsequent 24 hr (8 mg of phenobarbital per 100 g body weight).

The simultaneous appearance of labeled albumin in the two smooth subfractions during its intracellular transport indicates that both smooth I and smooth II microsomes derive either from the smooth ER or from related systems (10). It is difficult to assess the distribution of Golgi membranes at the present stage of study. Homogenization in 0.25 M sucrose, which is the method used in our experiments, converts the tubular profiles to vesicular structures, thus preventing identification. Further, no specific marker enzymes—at least for biochemical assay—are known for Golgi membranes. Therefore, the presence of Golgi vesicles in the smooth I and (or) smooth II subfractions cannot be excluded.

Published data on the PL composition of the total microsomal fraction (43, 44), ^{32}P incorporation (45, 46), cholesterol content (47–49), and TG content (50) are in agreement with the findings presented in this paper. The total microsomes differ markedly in lipid content and composition from other subcellular fractions of the liver. The high cholesterol content of the plasma membrane (49, 51), the presence of cardiolipin in the mitochondrial inner membrane (52) and lysosomes (53), the low cholesterol content of the outer mitochondrial membrane (54), and the low PL (55) and high TG (50) content of the nuclei have been described previously.

A large part of the microsomal protein does not originate in the membrane itself, and when effective washing procedures are employed, about 40% of the total protein can be removed. The resultant, relatively pure membranes of all three subfractions (rough, smooth I, and smooth II) have the same PL/protein ratio of about 0.40, and both the class and the amount of individual PL are similar in the membranes of the three subfractions. Using differential centrifugation, Manganiello and Phillips reported a similar PL composition of rough and total smooth membranes (56). On the other hand, the NL content is less homogeneous. The two major NL are present in high amounts in both smooth subfractions, which therefore exhibit high ratios of Ch/PL and TG/PL in comparison with those of the rough microsomes.

Furthermore, incorporation studies distinguish not only the rough microsomes from the smooth subfractions

but also the two smooth fractions from each other. The low rate of acetate-^{3}H incorporation into the Ch of the smooth I subfraction and the low rate of glycerol-^{3}H labeling of the total PL of the smooth II microsomes suggest that the turnover rates of lipids in the different microsomal membrane fractions are not identical. These findings may actually correspond to differences in the biosynthetic capacity of different membranes. Future analysis of the various enzymes and pathways of lipid synthesis, as well as studies of the half-lives of individual lipid components, might provide additional information on this aspect.

Experiments such as the washing procedures, DOC treatment, and glycerol-^{3}H incorporation studies strongly suggest that the presence of NL in microsomes is not the result of adsorption of cytoplasmic material. The available data may indicate that the NL of the microsomal membranes occur in two or more compartments, one of which would have a structural role and the other a metabolic role. The NL with a metabolic role might display more dynamic properties, such as a high turnover rate. The experimental observation that TG are synthesized within the endoplasmic reticulum and subsequently transported through the canal system, representing the very low density lipoproteins (57, 58) would favor such an interpretation. Furthermore, a part of the cholesterol which is the substrate for bile acid synthesis might also be localized in a metabolic compartment. In spite of these possibilities, it is probable that the differences in NL composition among subfractions reflect the amount of NL present in the structural compartment of the membranes. In agreement with such a structural role of the NL are the results of the phenobarbital treatment. Phenobarbital is known to increase the amount of endoplasmic membranes in the cell, probably by a complex mechanism which may include stimulated PL (39), cholesterol (59–61), and protein synthesis (62), as well as decreased breakdown (62, 63). In spite of the increase in the PL content (per g of liver) of rough and smooth I, but not smooth II microsomes after drug treatment, the NL/PL ratios remained the same in the three subfractions, which emphasizes the stability of the lipid composition of the microsomal membranes. DOC is known to solubilize a part of the microsomal membranes (18). In our experiments, 60–70% of both NL and PL remained in the attached state after DOC treatment, which again suggests that at least a large part of the NL is tightly bound to the membranes. The rate of incorporation of various precursors is higher in liver microsomes from fed animals than in starved animals (64). This finding is also in agreement with the theoretical existence of two cholesterol compartments—that of starvation chiefly representing a constituent with relatively low metabolic activity, and the increase after food supply probably reflecting a stimulation of bile acid synthesis.

The observed differences among the subfractions are surprising. The rough and smooth I microsomes contain an equal distribution of many, but most probably not all, constitutive enzymes (3–5). The half-lives of the proteins and of the PL of rough and total smooth microsomes are about the same (8), and experimental evidence suggests a transfer of membranes from the rough to the smooth compartment (6). This might lead one to assume the existence of identical membrane compositions for at least the rough and smooth I subfractions. Since this is not the case, two explanations may be proposed. First, the isolated microsomal subfractions are still heterogeneous. Experimental evidence supports this idea with regard to both rough (65) and total smooth microsomes (3, 66). The heterogeneity of the total smooth microsomes is also emphasized in this paper. The second possibility is that although the smooth microsomes are identical with the rough during the first stage of their biosynthesis, they may later undergo change in their composition and properties. Both the rough and total smooth microsomes seem to possess at least a part of the enzyme system for lecithin synthesis (67), and it is reasonable to assume that the microsomes, in conjunction with the soluble cytoplasm, are capable of synthesizing lipids both for endogenous and export purposes (68). Our experiments indicate that the biosynthetic capacities of rough and smooth membranes are different. However, a detailed investigation of the distribution of various enzymes that participate in lipid synthesis has not yet been carried out.

The reason for the high Ch/PL ratio in smooth membranes is not yet satisfactorily explained. De Bernard pointed out that in mixed films containing egg lecithin and cholesterol the cross-sectional area of the phospholipid molecules was reduced (69). Similar findings were also reported for synthetic lecithins and cholesterol (70). It is possible that cholesterol participates in compacting the lipid layers of biological membranes as well (71), and that its presence contributes to the stabilization of molecular arrangements. Cholesterol perhaps serves as a lipid organizer, permitting strong van der Waals interactions with other lipid molecules (72). Further experiments are required to decide whether or not there are corresponding differences in the physicochemical properties of rough and smooth membranes.

It has been well established that exchange of parts of the PL molecule, e.g. transacylations (73, 74), as well as exchange of serine (75, 76), choline (77), ethanolamine (78, 79), and inositol (80) without de novo synthesis occurs in microsomes. Omura et al. (8) concluded from half-life studies that the various parts of the microsomal PL molecule have different turnover rates. The incorporation rates of various PL precursors into liver microsomes after phenobarbital treatment show great

variation, including concurrent increase (81) and decrease (65). With these facts in mind, it is not surprising that the membranes of the various microsomal subfractions incorporate PL precursors at different rates.

Various studies have related the incorporation of labeled glycerol in the lipid extract to the phospholipid P of the same fraction. As can be seen from Fig. 3, this approach is not necessarily adequate. Even though liver microsomes contain only about 7% as much TG as phospholipid, the initial incorporation rate of glycerol into TG is 5–8 times that of the total PL fraction, and for this reason the TG contain 30–40% of the total microsomal radioactivity within the first 30 min after injection of labeled glycerol. Therefore, changes in cellular conditions that are predominantly due to the metabolically active TG might lead to a false interpretation of results if they are considered only in relation to the PL. When, therefore, the incorporation of labeled glycerol is studied, the NL and PL should be separated.

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