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ENZYMIC AND BIOCHEMICAL COMPOSITION OF SMOOTH AND ROUGH MICROSOMAL MEMBRANES FROM MONKEY, GUINEA PIG AND MOUSE LIVER

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Abstract—Smooth and rough microsomal membranes, prepared by density gradient centrifugation from livers of mice, guinea pigs and monkeys, were compared on the basis of biochemical composition, enzyme distribution, and fine structure. Phospholipid and cholesterol distribution varied considerably between smooth and rough membranes and between animal species. The following components of microsomal drug metabolism were studied: ethylmorphine demethylase, *l*-benzphetamine demethylase, aniline hydroxylase, NADPH cytochrome c reductase, cytochrome P-450, and spectral changes resulting from microsome–substrate interaction. Expressed per milligram of protein, all these components were concentrated about 2-fold in the smooth membranes of monkey and guinea pig liver, but were approximately evenly distributed between smooth and rough membranes of mouse liver. The results support the concept of heterogeneity among microsomal membranes. In addition to the well recognized species differences in microsomal drug metabolism, our data indicate important species differences in submicrosomal membrane composition and enzyme distribution.

THE MEMBRANOUS elements of the microsomal fraction of liver are of two morphologic types, rough and smooth, distinguished by the presence or absence of bound ribosomes (polysomes). Biochemically, hepatic microsomes are rich in ribonucleic acid (RNA), phospholipid and protein. Most (>95%) of the RNA is localized in the ribosomes, while the phospholipid is found exclusively in the membranous vesicles. Microsomal protein is about evenly distributed between ribosomes and vesicles.[1,2]

A number of investigations have established that smooth and rough microsomal membranes of liver differ somewhat in their enzymic composition. For example, glucose 6-phosphatase activity expressed per milligram of protein is 2- to 3-fold higher in rough membranes than smooth membranes of rat liver.[3–5] Similarly, the activity of UDP-glucuronyl transferase toward certain substrates is about twice as high in rough membranes as in smooth membranes of rabbit liver.[6]

Conversely, many of the microsomal enzymes that require NADPH and oxygen, utilize cytochrome P-450, and catalyze the metabolism of foreign compounds, are concentrated in smooth microsomal membranes.[7,8] The magnitude of this concentration varies somewhat with the substrate and the animal species investigated. For example, in rat liver microsomes, specific enzyme activities expressed as a ratio,

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smooth/rough, varied from 1·5 to 3·0 with different drug substrates, whereas with rabbit liver microsomes these ratios were as high as 4·0–5·0.[9,10] The relatively small differences in intramicrosomal enzyme distribution in rat liver could be explained by dilution of enzyme activity in rough membranes by enzymically inert ribosomal protein or by comparable differences in cytochrome P-450,[10] but the 4- to 5-fold differences in rabbit liver could not be accounted for on these grounds. Thus, enzyme activity expressed either per unit of phospholipid (i.e. "membrane") or per unit of cytochrome P-450 was significantly higher in smooth membranes than in rough.[10] This species difference raised a question as to whether rat or rabbit was the more typical animal species with regard to intramicrosomal enzyme distribution. In the present work, we examined the distribution of microsomal enzymes, electron transfer components, and other biochemical constituents in smooth and rough membranes of monkey, guinea pig and mouse liver. The results indicate that the drug-metabolizing capacity of monkey and guinea pig liver is concentrated in smooth membranes, but in mouse liver a distinctly different pattern was observed.

METHODS

Animals

Male albino Hartley guinea pigs (400–500 g), rhesus monkeys (2–3 kg) and albino Swiss mice (22–28 g) were obtained from NIH colonies and were maintained on standard laboratory diets and water until sacrifice. The animals were sacrificed by decapitation between 7 and 8 a.m. and livers were quickly removed and chilled in ice. Gall bladders were dissected free and discarded and the livers were minced with scissors. All subsequent tissue manipulations were performed at 0–4°.

Microsome fractionation

The procedure was that of Dallner[11] as recently modified for use in the Spinco 40·2 rotor.[12] Briefly, livers were homogenized in 0·25 M sucrose (25% homogenate) in a Potter-type homogenizer having a motor-driven plastic pestle. The homogenate was centrifuged at 10,000 *g* (av.) for 20 min. The 10,000 *g* supernatant was made 15 mM in CsCl, layered over 1·3 M sucrose containing 15 mM CsCl, and the gradients were centrifuged in a 40·2 rotor at 102,000 *g* (av.) for 120 min in a Spinco model L2-50 ultracentrifuge. Rough microsomal membranes penetrated through the 1·3 M sucrose layer to form a pellet at the bottom of the centrifuge tube, whereas the smooth membranes accumulated as a band at the interphase. Smooth membranes were transferred to another tube, diluted 2- to 3-fold with 0·15 M KCl, and were sedimented by centrifugation at 105,000 *g* for 75 min. The "intermediate" phase[12] was discarded. Whole microsomes were prepared by centrifuging a portion of the 10,000 *g* supernatant (containing 15 mM CsCl) at 105,000 *g* for 75 min in a Spinco No. 40 rotor. Microsomal membranes were resuspended in 0·15 M KCl–0·02 M tris–HCl prior to enzyme assays and other biochemical determinations.

Enzyme assays

Incubations were conducted aerobically at 37° for 10 min with shaking. Microsomal ethylmorphine demethylase, *l*-benzphetamine demethylase and aniline hydroxylase activities were determined in reaction mixtures having the following composition:

tris–HCl, pH 7·4, 120 mM; an NADPH-generating system consisting of NADP, 1·2 mM, $MgCl_2$, 10 mM; glucose 6-phosphate, 20 mM; and 2 units of glucose 6-phosphate dehydrogenase (yeast); substrate; microsomal protein (0·5–2·0 mg/ml); and sufficient 0·15 M KCl to bring the volume to 2·5 ml. In determinations of UDP-glucuronyl transferase activity with *p*-nitrophenol as the acceptor, the NADPH-generating system was replaced by UDPGA, 2 mM. Substrate concentrations were as follows: ethylmorphine, 30 mM; *l*-benzphetamine, 20 mM; aniline, 10 mM; *p*-nitrophenol, 0·4 mM. Preliminary experiments showed that enzyme activities were linear with time and with protein concentration under these conditions and that NADPH and the drug substrates were present in saturating concentrations.

NADPH cytochrome c reductase activity was measured at 37° by the method of Williams and Kamin[13] as described by Gigon *et al.*[14]

Analytical procedures

The *N*-demethylation of ethylmorphine and *l*-benzphetamine were estimated by assay of liberated formaldehyde,[10,15] aniline hydroxylase by formation of *p*-aminophenol,[16,17] and glucuronyl transferase activity by disappearance of the substrate.[6,18]

Microsomal protein was measured by the method of Lowry *et al.*[19] and RNA was determined by the orcinol reaction.[20,21] Lipids were extracted into chloroform–methanol (2:1, v/v) and the extract was washed with 0·88% KCl.[22] After digestion of an aliquot of the lipid extract with sulfuric and perchloric acids,[23] phospholipid phosphorus (PLP) was estimated as described by Chen *et al.*[24] Total cholesterol was determined in an aliquot of the lipid extract after alkaline hydrolysis of esters[25] by the Lieberman–Burchard reaction.[26] Microsomal cytochrome P-450 was determined by its carbon monoxide difference spectrum after reduction with dithionite.[10,27] Spectral changes produced by the addition of aniline or *l*-benzphetamine to microsomal suspensions were studied over a range of substrate concentrations from 0·5 to 5 mM. Spectral changes and cytochrome P-450 levels were determined with an Aminco–Chance split-beam spectrophotometer.

The data presented in the tables represent mean values from four (monkey and guinea pig) or five (mouse) experiments. Data were analyzed statistically by the Student *t*-test; the level of significance was $P < 0{\cdot}05$.

Electron microscopy

Microsomal subfractions, as pellets, were placed in Dalton's solution (OsO_4) and cut into ~1 mm cubes with a razor blade. After 60 min at 0–4°, the Dalton's solution was replaced by 0·5% uranyl acetate and the samples were allowed to stand overnight. The tissue was dehydrated in graded solutions of ethanol and propylene oxide and embedded in Epon-Araldite. Thin sections were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in an RCA EMU-3G electron microscope.

RESULTS

Biochemical composition of smooth and rough microsomal membranes

The distribution of protein, RNA, phospholipid and cholesterol (expressed per gram of liver) differed somewhat between smooth and rough membranes and rather

strikingly between animal species (Table 1). It is noteworthy that in mouse liver, protein and phospholipid were highly concentrated (4- to 5-fold) in rough microsomal membranes, whereas in monkey liver, phospholipid and cholesterol tended to be localized (2- to 3-fold) in smooth membranes.

Attention is drawn to the remarkably low yields of protein and phospholipid in the smooth membrane fraction of mouse liver (Table 1) relative to the rough membrane fraction or to the smooth membrane fractions of the other species. This peculiarity of mouse liver could not be explained by preferential loss of smooth vesicles during microsomal fractionation: the relative microsomal recoveries (smooth plus rough/total) of protein and phospholipid were comparable in the three species.

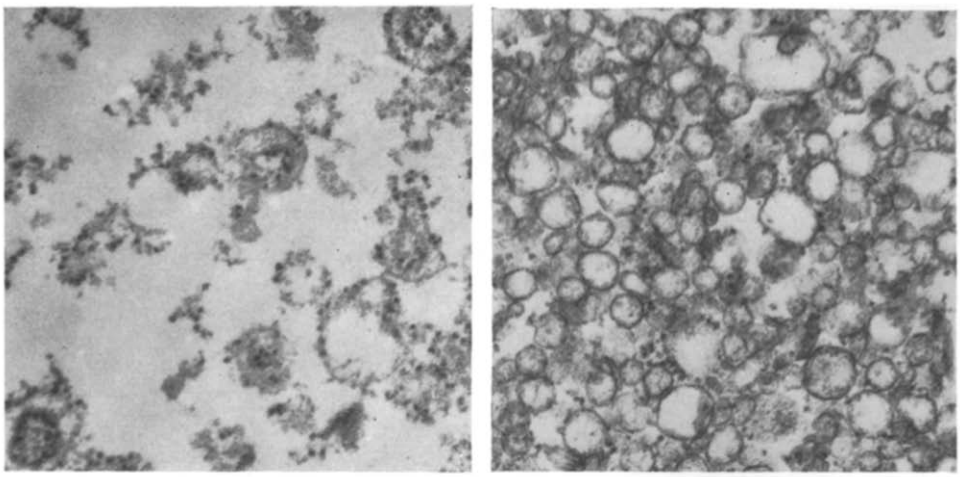
In accord with previous findings,[1,2] RNA expressed per milligram of protein was concentrated in the rough membrane fraction (Table 2) by a factor of 2·9 in the monkey, 3·1 in guinea pig and 2·2 in mouse. In monkey and guinea pig liver, the RNA/protein ratios were remarkably similar in the smooth membrane fractions and in the rough membrane fractions (Table 2), but this pattern was not observed in mouse liver. In all three species, phospholipid/protein ratios were somewhat higher in smooth membranes than in rough, which probably represents dilution by nonmembrane (ribosomal) protein in the rough membrane fraction. The ratio of cholesterol to phospholipid in smooth membranes of mouse liver was nearly four times that in rough membranes (Table 2). This is in agreement with recent findings[28,29] that smooth microsomal membranes of rat liver are several-fold richer in cholesterol than are rough membranes. In contrast, cholesterol was not highly concentrated in either microsomal subfractions from monkey or guinea pig liver (Table 2).

Electron microscopy

Rough membrane fractions from monkey (Fig. 1A) and guinea pig liver (Fig. 2A) consisted primarily of rough-surfaced vesicles and some free ribosomes, whereas smooth membrane fractions from these species (Figs. 1B, 2B) were composed of smooth vesicles contaminated to a minor extent with free ribosomes. The smooth vesicles varied somewhat in diameter (Figs. 1B, 2B). The rough membrane fraction of mouse liver (Fig. 3A) was composed of rough vesicles and free ribosomes; the rough vesicles appeared to have a higher complement of ribosomes than corresponding fractions from monkey or guinea pig. The smooth membrane fraction from mouse liver (Fig. 3B) was unusual morphologically as well as biochemically (Tables 1, 2) and enzymically (see below). Although smooth vesicles predominated and rough vesicles were relatively rare, much of the field was occupied by amorphous electron-dense particles. The finding that RNA was less restricted to the rough membrane fraction of mouse liver (Table 2) suggests greater contamination of smooth membrane fraction with RNA, presumably ribosomal in origin, in this species than in monkey or guinea pig.

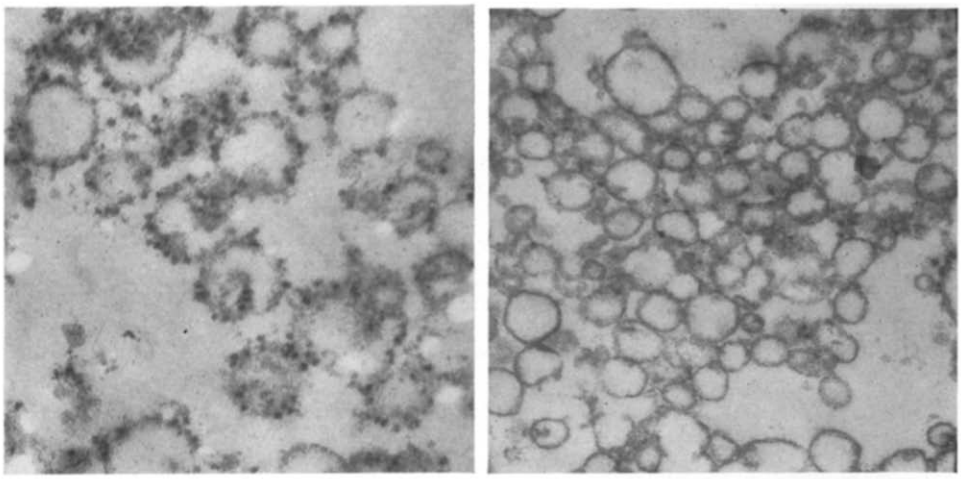
Distribution of components of microsomal electron transfer and drug metabolism in smooth and rough membranes

In monkey and guinea pig liver, NADPH cytochrome c reductase and cytochrome P-450 were concentrated about 2-fold in smooth membranes (Table 3). Similarly, the maximal spectral change elicited by addition of aniline or *l*-benzphetamine to microsomal fractions was greater in smooth than in rough membranes. These spectral



(a) (b)

FIG. 1. Electron micrographs of microsomal subfractions from monkey liver. The magnification in Figs. 1–3 is 22,000 ×. (a) rough membranes; (b) smooth membranes.



(a) (b)

FIG. 2. Electron micrographs of microsomal subfractions from guinea pig liver. (a) rough membranes; (b) smooth membranes.

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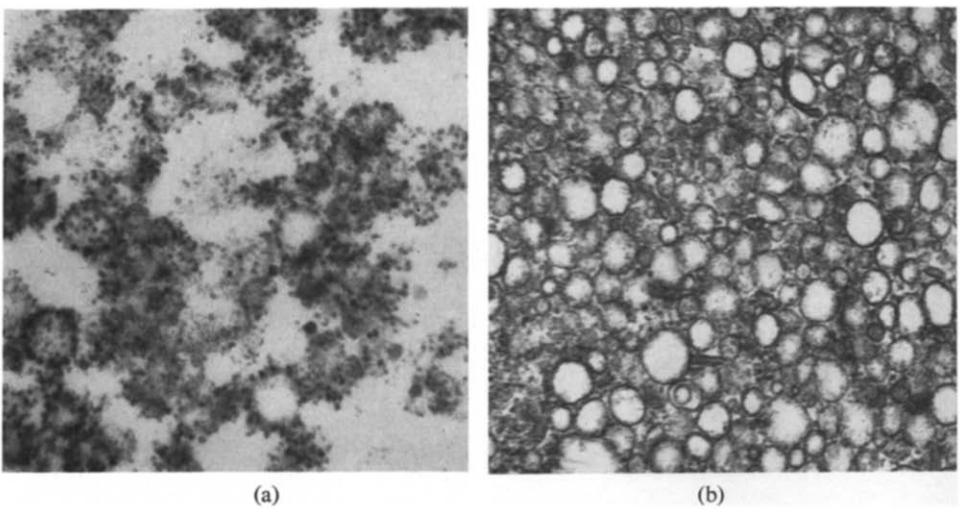

FIG. 3. Electron micrographs of microsomal subfractions from mouse liver. (a) rough membranes; (b) smooth membranes.

TABLE 1. BIOCHEMICAL COMPOSITION OF SMOOTH AND ROUGH MICROSOMAL MEMBRANES OF LIVER

	Monkey			Guinea pig			Mouse		
	Whole	Smooth	Rough	Whole	Smooth	Rough	Whole	Smooth	Rough
Protein (mg/g liver)	24·4 ±4·5	8·8* ±0·9	7·0* ±0·4	21·1 ±1·1	5·9* ±0·9	10·6* ±1·0	16·4 ±2·2	2·0* ±0·2	10·2* ±1·4
RNA(mg/g liver)	1·93 ±0·12	0·41* ±0·08	0·95* ±0·09	2·21 ±0·43	0·27* ±0·04	1·51* ±0·07	2·65 ±0·18	0·18* ±0·04	2·03* ±0·20
Phospholipid(μg PLP/g liver)	311 ±69	182* ±17	83* ±13	327 ±38	130 ±18	137 ±20	212 ±42	35* ±3	155* ±48
Cholesterol (mg/g liver)	0·44 ±0·13	0·28* ±0·03	0·09* ±0·01	0·36 ±0·11	0·14 ±0·07	0·14 ±0·05	0·34 ±0·05	0·13 ±0·03	0·15 ±0·04

* Significant difference (smooth versus rough) at $P < 0·05$.

TABLE 2. DISTRIBUTION OF RNA, PROTEIN, PHOSPHOLIPID AND CHOLESTEROL AMONG HEPATIC MICROSOMAL SUBFRACTIONS

	Monkey			Guinea pig			Mouse		
	Whole	Smooth	Rough	Whole	Smooth	Rough	Whole	Smooth	Rough
RNA/Protein	0·079	0·047	0·136	0·105	0·046	0·142	0·101	0·090	0·199
Phospholipid*/Protein	0·013	0·021	0·012	0·015	0·022	0·013	0·013	0·018	0·015
Cholesterol/Phospholipid*	1·41	1·54	1·09	1·10	1·08	1·02	2·55	3·71	0·97

* As phospholipid phosphorus (PLP).

TABLE 3. DISTRIBUTION OF NADPH CYTOCHROME C REDUCTASE, CYTOCHROME P-450 AND MAXIMAL SPECTRAL CHANGES IN HEPATIC MICROSOMAL SUBFRACTIONS

	Monkey			Guinea pig			Mouse		
	Whole	Smooth	Rough	Whole	Smooth	Rough	Whole	Smooth	Rough
NADPH cytochrome c reductase (nmoles cytochrome c reduced/mg protein/min)	133 ±17	183* ±15	83* ±28	187 ±20	254* ±39	141* ±22	190 ±44	172 ±38	194 ±41
Cytochrome P-450 ($O.D._{450-490}$/mg protein)	0·071 ±0·012	0·085* ±0·008	0·044* ±0·008	0·091 ±0·007	0·132* ±0·025	0·036* ±0·014	0·052 ±0·016	0·046 ±0·009	0·055 ±0·016
Maximal spectral change ($\Delta O.D.$/mg protein 10^3) aniline	26±2	31*±8	19*±6	16±5	24*±6	16*±2	19±6	14±2	15±2
l-Benzphetamine	39±7	49*±16	23*±4	23±4	32*±6	18*±4	25±3	25±12	23±8

* Significant difference (smooth versus rough) at $P < 0·05$.

TABLE 4. SUBMICROSOMAL DISTRIBUTION OF SOME HEPATIC ENZYME ACTIVITIES

	Monkey			Guinea pig			Mouse		
	Whole	Smooth	Rough	Whole	Smooth	Rough	Whole	Smooth	Rough
Ethylmorpine demethylase	31·2 ±9·0	40·2* ±12·7	21·3* ±8·4	16·0 ±2·8	22·1* ±3·2	12·9* ±2·3	51·1 ±5·7	43·2 ±13·7	55·1 ±7·7
l-Benzphetamine demethylase	18·4 ±3·6	23·6* ±6·2	12·8* ±3·2	12·1 ±2·4	18·1* ±1·2	9·4* ±1·2	21·4 ±4·1	18·8 ±7·6	22·1 ±3·8
Aniline hydroxylase	2·5 ±0·6	3·2* ±0·7	1·5* ±0·3	3·2 ±0·6	4·4* ±0·3	2·8* ±0·4	6·0 ±1·8	4·5 ±2·5	6·5 ±1·1
UDP-glucuronyl transferase	10·7 ±3·4	10·1* ±2·7	20·1* ±2·8	12·1 ±1·4	18·7 ±1·9	26·4 ±8·3	6·3 ±3·2	5·9 ±3·7	6·4 ±3·0

* Significant difference (smooth versus rough) at $P < 0.05$.

TABLE 5. DISTRIBUTION OF COMPONENTS OF MICROSOMAL ELECTRON TRANSPORT AND DRUG METABOLISM IN MICROSOMAL SUBFRACTIONS*

	Monkey (Smooth/Rough)	Guinea Pig (Smooth/Rough)	Mouse (Smooth/Rough)
NADPH cytochrome c reductase	2·2	1·8	0·9
Cytochrome P-450	1·9	3·7	0·8
Max. spectral change (Aniline)	1·6	1·5	0·9
Max. spectral change (*l*-Benzphetamine)	2·1	1·8	1·1
Ethylmorphine demethylase	1·9	1·7	0·8
l-Benzphetamine demethylase	1·8	1·9	0·9
Aniline hydroxylase	2·1	1·6	0·7
Glucuronyl transferase	0·5	0·7	0·9

* Values represent the distribution of components (smooth/rough) calculated per milligram of microsomal protein.

changes are thought to represent combination of the drug substrate with the oxidized form of cytochrome P-450.[30,31] In mouse liver, however, NADPH cytochrome c reductase, cytochrome P-450, and maximal spectral changes were evenly distributed between smooth and rough membranes (Table 3).

In monkey and guinea pig liver, the specific activities of ethylmorphine and *l*-benzphetamine *N*-demethylases and aniline hydroxylase were significantly higher (~2-fold) in smooth than in rough membranes (Table 4). By contrast, UDP-glucuronyl transferase activity was significantly higher in the rough membranes of monkey liver and, though not statistically significant, tended to localize in rough membranes of guinea pig liver. Again, the mouse was atypical in that none of the enzyme activities examined were concentrated in either fraction.

A general résumé of the distribution of components of microsomal drug metabolism between smooth and rough membranes is found in Table 5.

DISCUSSION

A current conception of the electron transfer system of hepatic microsomes which is involved in the metabolism of foreign chemical compounds is presented in Fig. 4. It is of interest that in monkey and guinea pig liver, all known components of this

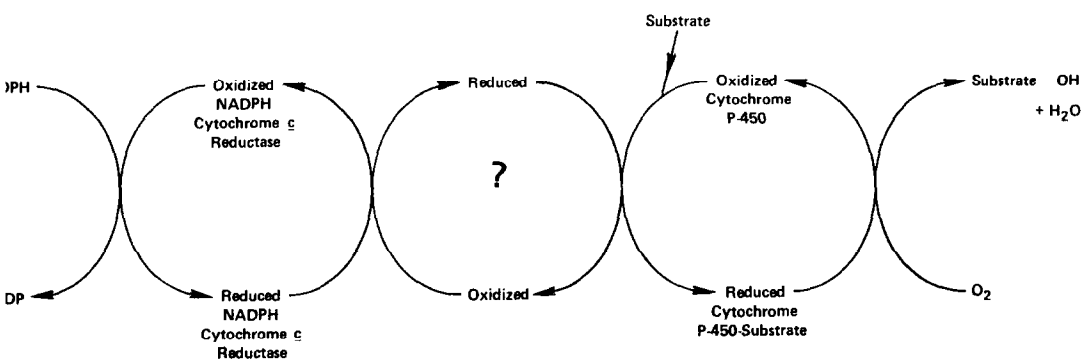

FIG. 4. A current conception of microsomal electron transfer.

system were concentrated to about the same degree (~2:1) in smooth microsomal membranes (Table 5). These differences were due, in part, to ribosomal protein dilution in the rough membrane fraction. If the enzyme activities were expressed per unit of phospholipid rather than per unit of protein, many differences between smooth and rough membranes disappeared.

In mouse liver, none of the components of the drug-metabolizing system were significantly localized in either membrane fraction. These findings distinguish the mouse from the monkey and guinea pig (Tables 1–4), the rat, and rabbit.[9] In terms of microsomal drug metabolism, mice exhibit certain other peculiarities. For example, exposure to the insecticide DDT results in microsomal enzyme induction in virtually all animal species that have been examined, but is without effect in mice.[32] Moreover, in rats there is a pronounced sex difference in drug metabolism; microsomes from male rats catalyze the oxidation of hexobarbital and aminopyrine about three times as rapidly as microsomes from female rats.[16] However, in mice, a sex difference in drug metabolism is present in some strains but not in others and, when a sex difference is present, the higher enzyme activities are found in the female.[33]

It has been suggested that cholesterol is unevenly distributed between smooth and rough microsomal membranes. For example, in rat liver, cholesterol expressed per unit of membrane (phospholipid) was several-fold richer in smooth membranes than in rough.[28,29,34] On the other hand, it has recently been proposed[35] that the endoplasmic reticulum of rat liver contains no endogenous cholesterol and that the cholesterol found in the microsomal fraction arises from contamination by plasma membrane or other organelles. Although our data do not address this question, they indicate (Table 2) that cholesterol–phospholipid ratios in smooth and rough microsomal membrane fractions vary widely among different animal species.

Previous work has shown that, although the phospholipid composition of smooth and rough microsomal membranes is nearly identical,[29] their protein composition differs. After removal of ribosomal contamination, the protein components of smooth and rough membranes of rat liver were separated by electrophoresis.[36] Each microsomal fraction contained several unique proteins; in addition, smooth and rough membranes contained a number of similar proteins whose relative distribution differed markedly.

After the injection of [^{3}H]-cortisol to rats, unchanged steroid accumulated in about a 5-fold higher concentration in smooth microsomal membranes of liver than in rough membranes. Electrophoretic analysis revealed the presence of four anionic metabolites in addition to cortisol in smooth membranes, but only unchanged steroid in rough membranes.[37]

Recent reports support the proposition of enzymic heterogeneity in microsomal membranes obtained from diverse biological sources. For example, Inano *et al.*,[38] studied the submicrosomal distribution of certain enzymes involved in steroid biosynthesis in the adrenal cortex. Steroid 21-hydroxylase, 17-α-hydroxylase, and cytochrome P-450 were 10- to 100-fold more concentrated in smooth membranes (expressed on a protein basis) than in rough membranes. A recent report described an NADPH, oxygen and cytochrome P-450-dependent system in the microsomes of the cotton leaf that catalyzes the *N*-demethylation of a herbicidal compound. Fractionation of the cotton leaf microsomes revealed that the specific activity of the *N*-demethylase was 3- to 6-fold higher in smooth membranes than in rough.[39]

Species differences in microsomal drug metabolism are widely recognized. These differences may be quantitative, presumably reflecting differences in enzymatic reaction rates, or qualitative, in which different biochemical pathways are involved.[40] Results obtained in the present investigation emphasize analogous species differences, quantitative and qualitative, in biochemical composition and enzyme distribution in microsomal subfractions.

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