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FURTHER STUDIES ON THE SUBMICROSOMAL DISTRIBUTION OF DRUG-METABOLIZING COMPONENTS IN LIVER

LOCALIZATION IN FRACTIONS OF SMOOTH MICROSOMES

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Abstract—Hepatic microsomes from male guinea pigs were fractionated into three populations—rough, smooth I (S-I) and smooth II (S-II)—by centrifugation in discontinuous sucrose gradients in the presence of Cs^+ and Mg^{2+}. The specific activities of several components of the microsomal drug-metabolizing system were unevenly distributed among the three fractions. Activities in smooth membranes were two to three times higher than in rough membranes. Comparison of the two smooth membrane fractions revealed a statistically significant concentration of drug-metabolizing components in S-II microsomes. Thus, ethylmorphine demethylase, aniline hydroxylase, NADPH-cytochrome *c* reductase, and cytochrome P-450, all expressed per milligram of protein, were 20–30 per cent higher in S-II than in S-I membranes. Morphologic differences between the two smooth membrane fractions were revealed by electron microscopy. Pretreatment of guinea pigs with phenobarbital for 4 days increased the specific activities of ethylmorphine demethylase, aniline hydroxylase, NADPH-cytochrome *c* reductase, and cytochrome P-450. The increase for each parameter was of approximately the same magnitude in each of the three microsomal fractions, but the four parameters increased disproportionately. Phenobarbital treatment produced significant increases in protein and phospholipid (per gram of liver) in S-I microsomes without accompanying changes in S-II or rough microsomes.

HEPATIC microsomal membranes are of two general classes, rough and smooth, distinguished by the presence or absence of bound ribosomes (polysomes). These classes differ in morphology, biochemical composition and certain enzyme activities.[1,2] Moreover, rough and smooth membranes are themselves heterogeneous. For example, centrifugation of rough microsomal membranes in a continuous sucrose gradient reveals uneven distribution of certain membrane components, enzyme activities and protein.[3,4] Similar results have been obtained with smooth membranes.[5,6]

Previous investigations have examined the distribution of drug-metabolizing components in smooth and rough microsomal membrane fractions of mammalian liver. The present work extends these studies to subfractions of the smooth membranes.

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METHODS AND MATERIALS

Animals. Male Hartley guinea pigs (450–525 g) were allowed free access to laboratory chow, fresh vegetables and tap water. Phenobarbital (Na salt) was administered intraperitoneally at a dose of 80 mg/kg once daily for 4 days; controls received equivalent volumes of 0·9% NaCl. The last dose was administered approximately 24 hr prior to sacrifice. The animals were sacrificed by decapitation between 7 and 8 a.m., livers were removed, gall bladders were dissected free and discarded, and the remaining hepatic tissue was chilled in ice. All subsequent tissue manipulations were conducted 0–4°.

Microsomal preparation and fractionation. Livers were homogenized in 0·25 M sucrose (25% homogenate) and the homogenate was centrifuged at 10,000 *g* for 20 min. The 10,000 *g* supernatant was made 15 mM with respect to CsCl and layered over 1·30 M sucrose containing 15 mM CsCl. The gradients were centrifuged at 102,000 *g* for 120 min in a Spinco 40·2 rotor as described by Bergstrand and Dallner.[7] Under these conditions, rough microsomal membranes penetrate through the 1·30 M sucrose layer and form a loose pellet at the bottom of the centrifuge tube; smooth membranes accumulate as a band at the interphase. The "intermediate phase"[7] was discarded. The band of smooth membranes along with a small volume of the 1·30 sucrose was removed with a syringe, diluted with about 30 per cent its volume of 0·15 M KCl and made 7 mM in $MgCl_2$. This mixture was layered over 1·15 M sucrose–7 mM $MgCl_2$ and centrifuged in a Spinco 40·2 rotor at 102,000 *g* for 45 min, which resulted in the deposition of a pellet at the bottom of the tube (smooth I microsomes) and a well-defined band at the interphase (smooth II microsomes). The latter fraction was transferred to another tube, diluted 3- to 4-fold with 0·15 M KCl, and sedimented by centrifugation at 165,000 *g* for 75 min. Microsomal fractions were resuspended in 0·15 M KCl immediately prior to enzyme assays.

Enzyme assays and analytical procedures. For the assay of ethylmorphine demethylase and aniline hydroxylase activities, incubation mixtures consisting of tris–HCl, pH 7·4 (120 mM), microsomal protein (1 mg/ml, final concentration), substrate, an NADPH-generating system consisting of NADP (1·2 mM), $MgCl_2$ (10 mM), glucose 6-phosphate (20 mM), and glucose 6-phosphate dehydrogenase (yeast, 2 units), and sufficient 0·15 M KCl to make a final volume of 2·5 ml were incubated for 10 min at 37° in air. Substrate concentrations were ethylmorphine, 30 mM, or aniline, 10 mM. Under these conditions, enzyme activities were zero-order with respect to substrate and NADPH concentration, and directly proportional to incubation time and protein concentration. NADPH-cytochrome *c* reductase activity was measured at 37° by the method of Williams and Kamin,[8] as described by Gigon *et al.*[9]

The rate of demethylation of ethylmorphine was estimated by assay of liberated formaldehyde,[10,11] and the hydroxylation of aniline by formation of *p*-aminophenol.[12,13] All determinations of ethylmorphine demethylation were corrected for "incubated blank", that is, apparent formaldehyde formed in the absence of ethylmorphine.[14] The incubated blank values differed considerably among microsomal subfractions; values obtained with smooth II microsomes were consistently about twice those obtained with smooth I microsomes.

Microsomal protein was estimated as described by Lowry *et al.*[15] with bovine serum albumin as the standard. For the assay of phospholipids, microsomal suspensions

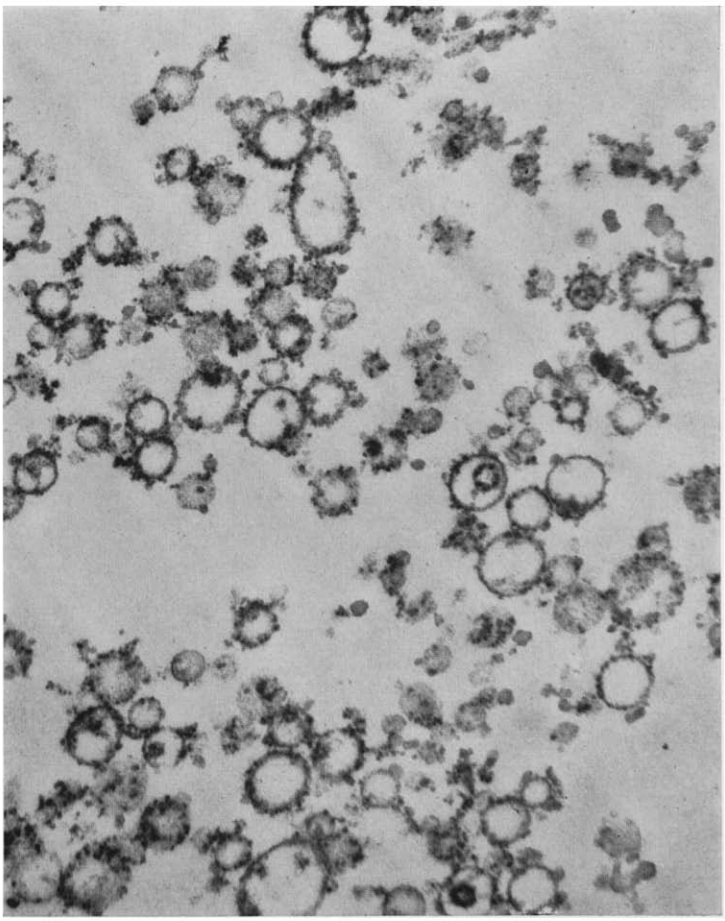

FIG. 1. Electron micrograph of the rough microsomal fraction prepared from guinea pig liver (× 22,000). The fraction consists primarily of rough-surfaced vesicles along with some free ribosomes or polysomes.

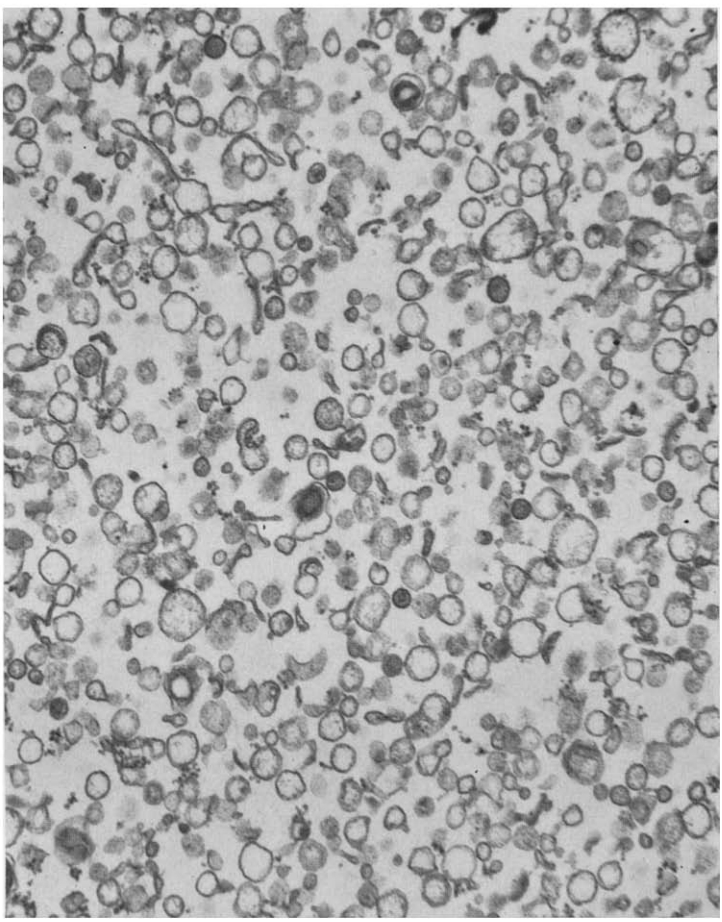

FIG. 2. Electron micrograph of the smooth I microsomal fraction prepared from guinea pig liver (× 22,000). Attention is directed to the heterogeneity, in shape and in intravesicular material, of the elements comprising this fraction. Round vesicles are most common; also evident are round profiles with projections, tubular or cylindrical structures, semilunar structures, and units consisting of two round vesicles connected by a narrow tube (resembling a dumbbell). The lumens of the vesicles range from highly electron dense (dark), through several grades of finely granular material, to light or transparent.

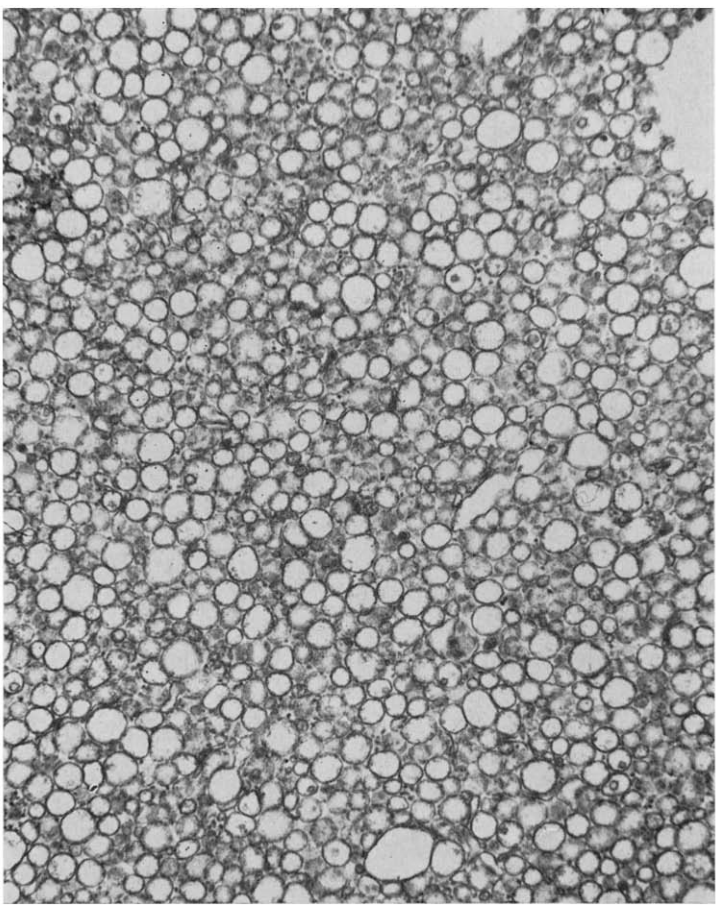

FIG. 3. Electron micrograph of the smooth II microsomal fraction prepared from guinea pig liver (× 22,000). Round vesicles, apparently deficient in electron-dense material, are the predominant structure in this fraction.

were extracted with 10 vol. chloroform–methanol (2:1, v/v) and the organic extract was washed with 0·88% KCl.[16] An aliquot of the washed extract was combusted with sulfuric and perchloric acids,[17] and the inorganic phosphorus (phospholipid phosphorus) was estimated as described by Chen *et al.*[18] Microsomal cytochrome P-450 was measured as its carbon monoxide complex after reduction with dithionite.[11,19] Statistical comparisons utilized the Student *t*-test at a significance level of $P < 0{\cdot}05$.

Electron microscopy. Microsomal pellets were placed in Dalton's solution[20] and quickly cut into small cubes (~1 mm) with a razor blade. After 60 min at 0–4°, the Dalton's solution was replaced by 0·05% uranyl acetate and the samples were allowed to stand overnight. The tissue was dehydrated in graded solutions to 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 with an RCA EMU-3G electron microscope.[21]

RESULTS

Electron microscopy of microsomal subfractions. The rough membrane fraction of guinea pig liver was composed primarily of vesicles bearing attached ribosomes (polysomes) and some aggregates of free ribosomes (Fig. 1).

Comparison of smooth I and smooth II fractions of guinea pig liver (Figs. 2 and 3) revealed certain morphologic differences. In general, the smooth II membrane fraction was more homogeneous than the smooth I fraction. For example, whereas the smooth II fraction consisted almost exclusively of round profiles. the smooth I fraction was a mixture of round profiles, round profiles with projections, and flattened tubular structures. The flattened, lunar-shaped structures suggest the presence of Golgi apparatus in the smooth I fraction.[22] Moreover, whereas the lumens of the smooth II vesicles were relatively deficient in electron-dense material, vesicles of the smooth I fraction ranged in content from highly dense osmiophilic material, through several grades of finely granular material, to an apparent lack of intravesicular material.

Enzyme levels in microsomal subfractions. In accord with earlier results in rats and rabbits,[11,23] the specific activities of the enzyme systems that catalyze the metabolism of ethylmorphine and aniline were higher in smooth membranes than in rough membranes of guinea pig liver (Table 1). Moreover, ethylmorphine demethylase and aniline hydroxylase activities, expressed per milligram of protein, were significantly higher (~30 per cent) in smooth II than in smooth I microsomes (Table 1). Similar distribution patterns were observed for NADPH-cytochrome *c* reductase and cytochrome P-450 (Table 2, control values); activities in smooth II microsomes were significantly higher (20–30 per cent) than in the smooth I fraction.

The ethylmorphine demethylase activity in smooth membrane subfractions correlated with the distribution of phospholipid. Accordingly, when expressed on a phospholipid basis, demethylase activities in smooth I and smooth II fractions did not differ significantly (Table 1). However, aniline hydroxylase activity, whether expressed on a protein or a phospholipid basis, was significantly higher (25–30 per cent) in smooth II than in smooth I microsomes (Table 1).

Effect of phenobarbital treatment on microsomal subfractions. Treatment of guinea pigs with phenobarbital for 4 days evoked significant increases in enzyme activities and cytochrome P-450 in all three microsomal fractions. Activities of ethylmorphine demethylase and aniline hydroxylase were increased by approximately 200 per cent

TABLE 1. DISTRIBUTION OF ETHYLMORPHINE DEMETHYLASE AND ANILINE HYDROXYLASE ACTIVITIES IN SUBFRACTIONS OF GUINEA PIG LIVER MICROSOMES*

	Rough		Smooth I		Smooth II	
	per mg protein	per μg PLP	per mg protein	per μg PLP	per mg protein	per μg PLP
Ethylmorphine demethylase (nmoles formaldehyde formed/min)	10·8 ± 2·6	0·91 ± 0·10	18·0 ± 2·2†	0·82 ± 0·11	23·1 ± 2·1†,‡	0·97 ± 0·10
Aniline hydroxylase (nmoles *p*-aminophenol formed/min	2·7 ± 0·6	0·23 ± 0·02	4·7 ± 0·3†	0·21 ± 0·01	6·2 ± 1·0†,‡	0·26 ± 0·03‡

* Values represent mean ± S.D. ($n = 4$).
† Differs significantly ($P < 0·05$) from corresponding value in rough fraction.
‡ Differs significantly ($P < 0·05$) from corresponding value in smooth I fraction.

TABLE 2. EFFECT OF PHENOBARBITAL (Pb) TREATMENT ON THE DISTRIBUTION OF ENZYME ACTIVITIES AND CYTOCHROME P-450 IN SUBFRACTIONS OF GUINEA PIG LIVER MICROSOMES*

	Rough		Smooth I		Smooth II	
	Control	Pb	Control	Pb	Control	Pb
Ethylmorphine demethylase (nmoles formaldehyde formed/mg protein/min)	10·8 ± 2·6	34·6 ± 7·6†	18·0 ± 2·2‡	50·2 ± 17·5†	23·1 ± 2·1‡,§	66·8 ± 28·4†
Aniline hydroxylase (nmoles *p*-aminophenol formed/mg protein/min)	2·7 ± 0·6	7·7 ± 1·3†	4·7 ± 0·3‡	12·7 ± 3·5†	6·2 ± 1·0‡,§	17·1 ± 6·5†
NADPH-cytochrome *c* reductase (nmoles cytochrome *c* reduced/ mg protein/min)	106 ± 19	245 ± 51†	210 ± 5‡	395 ± 73†	269 ± 23†,§	513 ± 87†
Cytochrome P-450 ($\Delta_{450-490}$/ mg protein)	0·050 ± 0·013	0·077 ± 0·004†	0·089 ± 0·014‡	0·128 ± 0·021†	0·107 ± 0·007‡,§	0·157 ± 0·030†

* Values represent mean ± S.D. ($n = 4$).
† Differs significantly ($P < 0·05$) from corresponding control value.
‡ Differs significantly ($P < 0·05$) for corresponding value in rough fraction.
§ Differs significantly ($P < 0·05$) from corresponding value in smooth I fraction.

TABLE 3. EFFECT OF PHENOBARBITAL (Pb) TREATMENT ON THE DISTRIBUTION OF PROTEIN AND PHOSPHOLIPID IN SUBFRACTIONS OF GUINEA PIG LIVER MICROSOMES*

	Rough		Smooth I		Smooth II	
	Control	Pb	Control	Pb	Control	Pb
Protein (mg protein/g liver)	15·1 ± 4·0	16·7 ± 4·0	3·07 ± 0·12	4·96 ± 0·54†	3·99 ± 0·73	4·97 ± 1·26
Phospholipid (μg phospholipid phosphorus/g liver)	181 ± 68	220 ± 30	67 ± 5	103 ± 19†	95 ± 10	119 ± 24
Phospholipid (μg phospholipid phosphorus/mg protein)	11·8 ± 1·8	13·3 ± 1·8	22·0 ± 0·9	21·2 ± 2·4	24·2 ± 3·7	24·8 ± 4·5

* Values represent mean ± S.D. ($n = 4$).
† Differs significantly ($P < 0·05$) from corresponding control value.

and roughly equivalent increases occurred in the three fractions (Table 2). Seemingly less responsive to phenobarbital treatment, NADPH-cytochrome *c* reductase was increased approximately 100 per cent compared with controls, while cytochrome P-450 increased about 50 per cent in the three microsomal subfractions (Table 2).

In contrast, phenobarbital treatment produced selective effects on microsomal protein and phospholipid. Expressed per gram of liver, the protein and phospholipid levels were significantly increased in the smooth I fraction, but were unchanged in the rough and the smooth II fractions (Table 3). The relative increases in protein and phospholipid were comparable; as a result, the phospholipid/protein ratios in the three membrane fractions did not change as a result of phenobarbital treatment (Table 3).

DISCUSSION

The extensive work of Dallner *et al.*[2,3,5,24] has established the biochemical and enzymic heterogeneity of smooth and rough microsomal membranes of rat liver. It has been recently appreciated that smooth and rough membrane fractions are, themselves, heterogeneous. Analysis of smooth or rough membranes by centrifugation in continuous gradients has revealed striking localization of enzyme activities in certain regions of the gradient.

The mechanism by which Mg^{2+} facilitates fractionation of smooth microsomal membranes is not known. However, Dallner's[24] work with rat liver indicated that a major morphologic difference between smooth I and smooth II membrane fractions was vesicle size. Thus, his smooth I fraction (Mg^{2+}-binding) was composed of relatively large vesicles (100–300 nm diameter), whereas the smooth II fraction (Mg^{2+}-free) consisted of smaller vesicles having a mean diameter of about 50 nm. In guinea pig liver, however, there were no conspicuous differences in vesicle diameter between the two smooth membrane fractions, even though certain other morphologic differences were noted (Figs. 2 and 3).

Recent work has revealed important species differences in the distribution of the mixed function oxidase system between smooth microsomes and rough microsomes of liver.[11,23,25] Enzyme activities, expressed as a ratio of smooth/rough, were highest (~ 5) in rabbit, intermediate (~ 2) in rat, guinea pig and monkey, and lowest (< 1) in mouse liver. The present investigation suggests the existence of a species difference in enzyme distribution in subfractions of smooth membranes.

Dallner[24] examined the distribution of electron transport components in subfractions of smooth microsomes of rat liver. The activities of NADH- and NADPH-cytochrome *c* reductase were concentrated 3- to 5-fold in smooth I membranes relative to smooth II; cytochrome P-450 was about 30 per cent higher in smooth I membranes. More recent work by Glaumann *et al.*[26] confirmed and extended these findings with rat liver microsomes. Thus, relative to smooth II membranes, NADPH-cytochrome *c* reductase was concentrated in smooth I microsomes by nearly 4-fold, cytochrome P-450 by 30 per cent, NADPH-cytochrome P-450 reductase about 8-fold, and aminopyrine demethylase nearly 3-fold.

Our data obtained with guinea pig liver indicate a different pattern of enzyme distribution. Thus the specific activities of ethylmorphine demethylase, aniline hydroxylase, NADPH-cytochrome *c* reductase and cytochrome P-450 were slightly but significantly higher (~ 30 per cent) in smooth II than in smooth I membranes (Table 2). It is noteworthy that these relatively small differences were remarkably reproducible

over four separate experiments. This reproducibility implies that the disruption of the endoplasmic reticulum during homogenization may be determined by the physicochemical characteristics of a non-uniform membrane.

Treatment of guinea pigs with phenobarbital increased the specific activities of ethylmorphine demethylase, aniline hydroxylase, NADPH-cytochrome *c* reductase and cytochrome P-450 in rough, smooth I and smooth II membranes. The increases were of the same magnitude in each of the three membrane fractions but, in contrast to earlier results with rat liver,[27-29] the four parameters did not increase in a parallel fashion. Thus, phenobarbital elicited large increases in ethylmorphine demethylase and aniline hydroxylase (~200 per cent), a moderate increase in NADPH-cytochrome *c* reductase (~100 per cent) and a relatively small increase in cytochrome P-450 (~50 per cent).

Phenobarbital treatment of guinea pigs produced a selective increase in the levels of protein and phospholipid in the smooth I membrane fraction with no accompanying changes in smooth II or rough membranes. Small increases in the latter fractions were not statistically significant. Similarly, treatment of rats with phenobarbital increased the protein and phospholipid content of smooth I membranes without concomitant changes in smooth II.[26,30]

Finally, species differences in the metabolism of foreign compounds have been long recognized; these differences may be quantitative, qualitative, or both.[31] In the light of this knowledge, it is surprising that species differences have been practically ignored in investigations of the biochemical and enzymic nature of microsomal subfractions. Previous work in our laboratory[11,23,25] has emphasized the importance of species differences in the distribution of drug-metabolizing component between smooth and rough microsomal membranes. Moreover, the results of the present investigation taken together with those of Dallner *et al.*[24,26] indicate that the distribution of drug-metabolizing components in fractions of smooth microsomes is markedly species-dependent.

Note added in proof—In a recent publication, Glaumann [*Chem.-Biol. Inter.* **2**, 369 (1970)] suggested that the microsomal subfractionation technic of Dallner "requires modifications before applying it to subfractionation of guinea pig homogenate". Unfortunately, no evidence of any sort was provided in support of this assertion. Although we stipulate the possibility of a species difference, in the absence of supporting data Glaumann's claim is open to doubt. Moreover, since "microsomes" is an operational term, it would seem to follow that microsomal subfractions can also be defined in operational terms. Finally, in the absence of adequate biochemical or enzymic "markers", it is not clear by what criteria the "modifications" in technic were determined.

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