SUBFRACTIONATION OF SMOOTH MICROSOMES FROM RAT LIVER

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ABSTRACT

Total smooth microsomes from rat liver isolated on a Cs^+-containing sucrose gradient were concentrated and subsequently fractionated by zone centrifugation on a stabilizing sucrose gradient. The prerequisite for fractionation is to prepare total smooth microsomes in a nonaggregated condition, as well as to utilize a procedure which counteracts enzyme inactivation. The median equilibrium density of the various smooth microsomal vesicles ranges from 1.10 to 1.18. The phospholipid/protein ratio is identical in all subfractions, but cholesterol, on a PLP basis, is enriched in the subfractions with the highest sedimentation velocity. The enzyme distribution pattern reveals a pronounced heterogeneity. A number of NADH- and NADPH-oxidizing enzymes are concentrated in the upper part of the gradient and exhibit a certain degree of separation from G6Pase. Mg^{++}-ATPase and AMPase are enriched in the lower part of the gradient. No specific enrichment of newly synthesized NADPH–cytochrome *c* reductase activity occurs in any of the subfractions after phenobarbital treatment. These data demonstrate that smooth microsomes, by adequate fractionation procedure, can be separated into subfractious of heterogeneous composition.

INTRODUCTION

A large part of the endoplasmic reticulum (ER)[1] in the liver parenchymal cell consists of smooth-surfaced tubules and cisternae (1). These membranes are somewhat concentrated in certain areas of the cytoplasm and are often associated with a number of glycogen granules (2). The smooth ER is delimited from the rough variety from both a morphological and a functional point of view (cf. 3, 4). The attached ribosomes represent the morphological basis for protein synthesis, a function present only in rough-surfaced ER. Apart from ribosomes, smooth membranes differ from the rough variety in several respects. On a phospholipid (PLP) basis, the former contain almost twice as much cholesterol as the rough membranes (5, 6). Perhaps some of the enzymes participating in the glycoprotein synthesis are enriched in the smooth microsomes (7, 8), but on the other hand, enzymes of the NADH- and NADPH-electron transfer chain and the various phosphatases are evenly distributed in the two main compartments of rat liver microsomes, e.g. total rough and total smooth fractions (9). There are differences in the incorporation rate of precursors into the cholesterol and PLP of rough and smooth microsomes but, measured with labeled glycerol, the half-life

[1] *Abbreviations used in this paper are as follows:* AMP, adenosine monophosphate; ATP, adenosine triphosphate; cyt., cytochrome; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; IDP, inosine diphosphate; MAOase, monoamino oxidase; NADH and NADPH, reduced di- and triphosphopyridine nucleotide; NL, neutral lipids; NT, neotetrazolium; PCA, perchloric acid; PLM, plasma membranes; PLP, phospholipids; red., reductase; RNA, ribonucleic acid; TCA, trichloroacetic acid.

of the total PLP in the two types of membranes is similar (5, 6, 10). The newly synthesized enzymes of the newborn and drug-treated animals first appear in the rough compartment and are subsequently transferred to the smooth ER (11, 12).

This paper describes subfractionation of smooth microsomes from rat liver by using zone centrifugation on a stabilizing sucrose gradient. The marked heterogeneity in the distribution of enzymic components indicates the complex composition of smooth microsomal membranes. A preliminary report of a part of this work has appeared (13).

MATERIALS AND METHODS

Animals

Adult male albino rats weighing 150–250 g were used. The animals were starved 20 hr before sacrifice. When phenobarbital-treated rats were used, 8 mg of phenobarbital/100 g body weight was injected intraperitoneally in a single dose at indicated time points.

Fractionation

PREPARATION OF SMOOTH AND ROUGH MICROSOMES: Isolation of smooth and rough microsomes was performed as earlier (6, 9), and according to Rothschild with certain modifications (14). Minced livers were homogenized in 0.25 M sucrose by means of a Teflon-glass homogenizer. No buffer was included in any of the media used in this procedure. A 20% homogenate of liver was centrifuged at 10,000 *g* for 20 min, and the supernate was diluted to restore the original volume. 8 ml of this suspension was layered over 3 ml of 1.31 M sucrose and centrifuged at 105,000 *g* for 7 hr in a Christ Omega II ultracentrifuge (Martin Christ, Osterode am Harz, W-Germany), rotor 60, tube angle 34°. The upper 0.25 M sucrose phase was removed with a pipette provided with a rubber aspirator, and discarded. The smooth microsomes at the interphase were collected together with as little as possible of the 1.31 M sucrose.

SUBFRACTIONATION OF SMOOTH MICROSOMES: After isolation of smooth microsomes on a discontinuous Cs^+-containing sucrose gradient (6, 9), smooth fractions from three tubes were pooled and diluted dropwise under stirring to 9 ml with cold distilled water, layered over 2.5 ml of 1.4 M sucrose, and centrifuged for 30 min at 75,000 *g* (Christ Omega II ultracentrifuge, rotor 60). The upper phase was discarded and the fluffy interphase, made up of concentrated smooth microsomes, was collected and diluted slowly with water to a final sucrose concentration of about 8%. 4 ml of this suspension was layered over 22 ml of a continuous sucrose gradient ranging from 0.29 M to 0.73 M. The linear sucrose gradients were prepared with a mixing chamber (15). Subfractionation was performed in an SW 27 rotor (Christ Omega II ultracentrifuge) at 56,000 *g* for 75 min. Fractions were collected through a needle inserted near the bottom of the tube just above the edge of the pellet. Any remaining sucrose on the wall and at the bottom of the centrifuge tube was carefully removed with filter paper. The pellet was suspended in 0.25 M sucrose.

A scheme of the fractionation procedure is presented in Fig. 1.

Millipore Filtration

Concentrated total smooth microsomes were diluted with 2 volumes of 0.15 M sucrose and filtered by suction pump through a filter of either 0.30 μ or 0.45 μ (Millipore Corp., Bedford, Mass.). In the case of subfractions, 1 ml of undiluted suspension was placed on the filter and 3 ml of 0.25 M sucrose was used for additional washing. The filtrate was cencentrifuged at 105,000 *g* for 90 min and the protein content of the pellet was compared with the same fraction which was not filtered but pelleted.

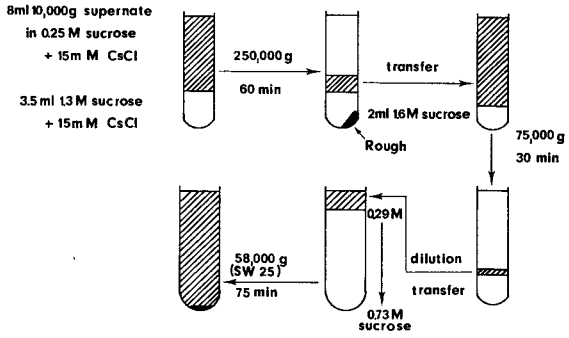

FIGURE 1 Schematic illustration of the subfractionation procedure for total smooth microsomes of rat liver.

Density Equilibrium Centrifugation

After subfractionation of total smooth microsomes, 3 ml of a chosen subfraction was layered on a continuous sucrose gradient of the appropriate sucrose range. Centrifugation to equilibrium was performed in an SW 27 rotor at 50,000 *g* for 16 hr. The position of the microsomes was determined by measuring the absorbance at 280 mμ after collection of fractions as described above. The linearity of the gradient after 16 hr centrifugation was tested by means of labeled sucrose-^{14}C.

Chemical Analysis

Protein was measured according to Lowry et al. with bovine serum albumin as standard (16). Ribonucleic acid (RNA) was determined as described earlier (6). For PLP analysis the pellets from different fractions were washed twice with cold 6% trichloroacetic acid (TCA) and once with distilled water in order to remove molecules containing polar phosphate. The lipids were extracted in chloroform-ethanol (2:1) in the presence of nitrogen and 0.05% butylated hydroxytoluene as antioxidant.

In order to separate neutral lipids from PLP, when determining the cholesterol content, the chloroform phase was passed through a silicic acid column, as has been described by Borgström (17). PLP and cholesterol were determined as earlier (6).

Enzyme Assays

The activities of NADH- and NADPH-cyt. *c* red., NADPH-neotetrazolium (NT) and NADH-ferricyanide red., as well as Mg^{++}-ATPase, G6Pase, IDPase, MAOase, and cytochrome *c* oxidase activities were estimated as before (5, 11). In the case of G6Pase and IDPase, 0.04% deoxycholate was included in the incubation medium (18).

Cytochrome b_5 and cytochrome P-450 were determined by difference spectroscopy. NADH was used to reduce cyt. b_5. This eliminated the error due to hemoglobin contamination which may arise on reduction with $Na_2S_2O_4$. For measurement of cyt. P-450, both cuvettes were reduced with $Na_2S_2O_4$ and the sample cuvette was bubbled with O_2-free CO. For determination of acid phosphatase, the fractions were frozen and thawed three times. The incubation medium contained 0.05 M Tris-acetate buffer, pH 5.0, 0.02 M β-glycerophosphate, 0.25% Triton X-100, and microsomes in a final volume of 1 ml. After incubation at 30°C for 20 min, the reaction was stopped with 1 ml of 1 N perchloric acid (PCA) and the liberated inorganic orthophosphate was measured by the Fiske-Subbarow method (19). The incubation medium for AMPase consisted of 0.1 M Tris-HCl buffer, pH 7.5, 0.01 M adenosine monophosphate (AMP), and 0.02 M $MgCl_2$. Incubation and measurement of PO_4^{--} were performed as described for acid phosphatase. Since sucrose inhibits AMPase and Mg^{++}-ATPase activities, corrections were made for each fraction by means of a standard curve (20).

Incorporation of Radioactive Substances

Glycerol-2-^{3}H (50 mCi/mmole) from the Radiochemical Centre, Amersham, Buckinghamshire, England, was diluted in sterile Ringer's solution. The rats were decapitated 10 min after interperitoneal injection (20 μCi/100 g). The liver was subfractionated, the lipids were extracted, and PLP and neutral lipids (NL) were separated as described above. Radioactivity was measured in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, Calif.), DPM-100, in a toluene scintillation mixture (21). DL-Mevalonic acid-2-^{3}H lactone (50 mCi/mmole, Radiochemical Centre) was injected intraperitoneally, 20 μCi/100 g. The subfractionation and separation of cholesterol from PLP were performed as described above.

RESULTS

Effect of Various Treatments

The results presented in Table I demonstrate the effect of various treatments on the smooth membranes estimated by Millipore filtration. Regardless of the method used to isolate total smooth microsomes, almost all microsomes pass the 0.45 μ and 75–80% the 0.30 μ filter, presupposing that the concentration is about 0.5 g/ml and the sucrose concentration is 0.6 M or higher. Pelleting and subsequent resuspension in 0.6 M sucrose causes almost complete aggregation which cannot be countered to any appreciable extent by addition of albumin to the suspension medium. The addition of CsCl to smooth microsomes has only minor effect, but a heavy aggregation occurs in the presence of $MgCl_2$. The importance of high protein and sucrose concentrations for the stabilization of smooth microsomes is illustrated in Table II. A low concentration of smooth microsomes suspended in a dilute sucrose solution, such as 0.15 M, results in aggregation. However, an increase of the protein concentration counteracts this undesirable effect to a great extent. Raising the sucrose concentration makes it possible to obtain a 80% pass of the vesicles through both 0.30 μ and 0.45 μ filters.

The above experiments clearly indicate that the maintaining of smooth microsomes in a non-

TABLE I

Effect of Various Treatments on Smooth Microsomes

Cs^+-smooth denotes microsomal fraction isolated on a discontinuous sucrose gradient containing CsCl (6, 9). In experiment 1, the interphase layer was sucked off and used for the experiment. In experiment 2, the interphase layer containing smooth microsomes was diluted with 0.25 M sucrose and sedimented at 105,000 *g* for 90 min. The pellet was resuspended in 0.25 M sucrose, 0.5 g/ml (wet weight of liver). When albumin was present, the suspension medium consisted of 0.25 M sucrose and 5% dialized bovine serum albumin. *R-smooth* denotes smooth microsomes isolated with the Rothschild procedure (14) but with modifications which together with the Millipore filtration procedure are described in Materials and Methods.

Experiment	Microsomes	Treatment	% of total protein in the filtrate	
			Filter size	
			0.30 μ	0.45 μ
1	Cs^+-smooth, 0.8 g/ml in 0.7 M sucrose	None	81	83
	"	10 mM $MgCl_2$	32	37
2	Cs^+-smooth, 0.5 g/ml in 0.7 M sucrose	Resuspension after pelleting	20	32
	"	"		
		+ 0.5% albumin	28	41
3	R-smooth, 0.5 g/ml in 0.6 M sucrose	None	76	83
	"	+ 15 mM CsCl	63	81
4	R-smooth, 1 g/ml in 0.6 M sucrose	Resuspension after pelleting	10	25
	"	"		
		+ 0.5% albumin	30	40

aggregated form requires high protein and sucrose concentrations as well as avoidance of pelleting.

Effect of Storage

The degree of stability of microsomal electron transport enzymes and phosphatases is demonstrated in Tables III and IV. Regardless of sucrose and protein concentration, both NADH- and NADPH-cyt. *c* red. activities of smooth and rough microsomes exhibit a pronounced stability within 20 hr of storage. The measurable amount of cyt. b_5 is somewhat decreased if the microsomes are suspended in distilled water, while cyt. P-450 displays a somewhat greater lability in the two subfractions. A good recovery of this latter enzyme can only be obtained in the presence of 0.88 M sucrose at a microsomal concentration of about 1 g/ml.

As regards phosphatases, ATPase, and, in particular, AMPase activities are constant during a storage period of 20 hr, while G6Pase is seriously inactivated after this duration of storage, if the conditions of high protein and sucrose concentration are not fulfilled. After a shorter period of storage, such as 3 hr, which corresponds to the actual time for the fractionation procedure utilized in this study, no larger inactivation occurred provided that the microsomes were suspended in sucrose solution. Additionally, the data in Tables III and IV show that no major differences exist between smooth and rough microsomes under the various conditions used.

Millipore Filtration

The smooth microsomal subfractions, after the fractionation procedure, were tested for aggregation on a millipore filter. 70–80% of the vesicles passed the 0.30 μ filter, which excludes a greater degree of aggregation. On the other hand, the microsomes in the pellet after the subfractionation

TABLE II

Influence of Protein and Sucrose Concentration on Smooth Microsomes

Cs⁺-smooth and *R-smooth* were prepared as in Table I. In both cases, the interphase fraction was taken and subsequently diluted with 0.15 M or 0.88 M sucrose as indicated. Millipore filtration and protein determination were performed as described in Materials and Methods.

Experiment	Microsomes	Sucrose, 0.15 M: Filter size 0.30 μ	Sucrose, 0.15 M: Filter size 0.45 μ	Sucrose, 0.88 M: Filter size 0.30 μ	Sucrose, 0.88 M: Filter size 0.45 μ
	g/ml	*% of total protein in the filtrate*			
1. Cs⁺-smooth					
	0.025			48	48
	0.05	5	19	50	64
	0.1	29	35	50	70
	0.2	54	64	68	75
	0.4	63	78	75	81
	0.8			81	80
2. R-smooth					
	0.03	13	34		
	0.06	28	38	38	40
	0.12	46	47	53	70
	0.24	52	63	65	78
	0.5			75	79

procedure remain on the filter, which indicates an almost complete aggregation.

Density

The subfractionation procedure for smooth microsomes is performed on a stabilizing gradient using the zone centrifugation principle, and so the separation depends to a large extent on the size and only to a minor extent on density differences (22, 23). Because of the osmotic response of the smooth vesicles (24), this size varies on a gradient and the determination of it is only of relative value. Equilibrium density is a good characteristic of subcellular particles, and the experimental findings of the density distribution of some subfractions are shown in Fig. 2. The gradient was divided into four fractions, which, together with the pellet, were centrifuged on a sucrose gradient to isopycnic equilibrium.

Calculating the median value of equilibrium density in aqueous sucrose (ρ_e), using the data in Fig. 2, gives values of 1.10–1.18 from top to bottom in increasing order of magnitude. These figures are in good agreement with those found by Rothschild for smooth microsomes (14).

Chemical Composition

The chemical composition of subfractions was determined after recentrifugation in order to remove the soluble protein (Table V). About 20% of the protein is recovered in the pellet and 40% in fractions 4 and 5, a distribution which is also valid for PLP. The PLP/protein ratio is very similar in all subfractions, ranging from 0.34 to 0.39. Cholesterol, which is present in relatively large amounts in total smooth microsomes, is most abundant in the pellet. On a PLP basis, the distribution is even on the gradient (9–11%) except for the pellet and fraction 1, which display a ratio of about 0.17. Small amounts of RNA are present in all subfractions.[2] The enrichment in fraction 4 is most probably due to slowly sedimenting free ribosomes.

Because of the limitation of the material in some of the individual fractions after subfractionation, protein was used to calculate specific enzyme activities. This is applicable only if the vast majority of protein in the different fractions is membranous, and so the amount of nonsedimentable protein, i.e. nonmicrosomal, was determined. When the pooled fractions from the gradient were subjected to centrifugation (105,000 *g* for 90 min), 10–15% of the total protein was found to remain in the supernate. Only that part of the gradient which corresponds to the original total smooth microsomes contains larger amounts of nonsedimentable protein. With this limitation in mind, it therefore seems justifiable to present enzyme activities or amounts on a protein basis.

Enzyme Distribution

ENZYME RECOVERY: The specific activities or amounts of enzymes in the total smooth fraction appear in Table VI. The same table also summarizes the recoveries from the gradient after centrifugation. These are in all cases 80% or higher.

PHOSPHATASES: The distribution of four phosphatases in smooth microsomes is shown in Fig. 3. Smooth microsomes contain high G6Pase and IDPase activities, two enzymes which are re-

[2] The presence of RNA in smooth microsomal membranes is much debated, since it can be due to either ribosomes, subunits of ribosomes, or "membrane constituents" (25–27).

TABLE III

Stability of Electron Transport Enzymes in Smooth and Rough Microsomes during Storage

Smooth and rough microsomal subfractions were prepared on a CsCl-containing sucrose gradient (6, 9). Separate pellets were resuspended in distilled water, 0.25 M or 0.88 M sucrose, in a concentration of 1 g/ml (wet weight of liver). After subsequent dilutions, enzyme activities or amounts were determined and used as control values. Storage was performed in an ice-water bath for 20–24 hr. The values are given as the per cent of control.

Experiment	Medium and microsomal concentration	NADH-cyt.*c* red.		NADPH-cyt.*c* red.		Cyt. b_5		Cyt. P-450	
		smooth	rough	smooth	rough	smooth	rough	smooth	rough
1	Distilled H_2O, 1 g/ml					97	77	87	70
	" 0.1 g/ml	130	125	107	106				
	" 0.05 g/ml					94	65	41	36
	" 0.01 g/ml	130	118	121	79				
2	0.25 M sucrose, 1 g/ml					83	94	82	88
	" 0.1 g/ml	134	130	103	116				
	" 0.05 g/ml					91	75	47	43
	" 0.01 g/ml	140	91	98	102				
3	0.88 M sucrose, 1 g/ml					105	105	91	91
	" 0.1 g/ml				124				
	" 0.05 g/ml					105	90	83	67
	" 0.01 g/ml	137	125	88	124				

TABLE IV

Stability of Phosphatases in Smooth and Rough Microsomes during Storage

Preparation and treatment of smooth and rough microsomal fractions as in Table III. The time of storage was 3 or 20 hr. The values are given as the per cent of control.

Experiment	Medium and microsomal concentration	ATPase		AMPase		G6Pase			
		smooth	rough	smooth	rough	smooth		rough	
		20 hr	20 hr	20 hr	20 hr	3 hr	20 hr	3 hr	20 hr
1	Distilled H_2O, 1 g/ml	82	82	120	140				
	" 0.1 g/ml	76	77	107	107	82	14	84	26
	" 0.05 g/ml	68	70			67	12	80	7
2	0.25 M sucrose, 1 g/ml	98		108	130				
	" 0.1 g/ml	81	85	110	115	107	47	110	32
	" 0.05 g/ml	81	83	106		85	28	105	10
3	0.88 M sucrose, 1 g/ml	87	83	121	145				
	" 0.1 g/ml	100	83	116	103	114	91	125	89
	" 0.05 g/ml	100				107	75	124	72

garded as microsomal (18). The other phosphatases measured here, AMPase and Mg^{++}-ATPase, are found in highest concentration in other subcellular elements(29). The criteria for gradient centrifugation were set up in order to prevent enzyme inactivation (see Table IV), which is apparent from the high recovery values, 80–90% in the case of phosphatases. The uneven distribution pattern, therefore, can be regarded as significant, a fact which is also apparent from values of standard deviation (not shown in Fig. 3).

AMPase and ATPase activities, which closely

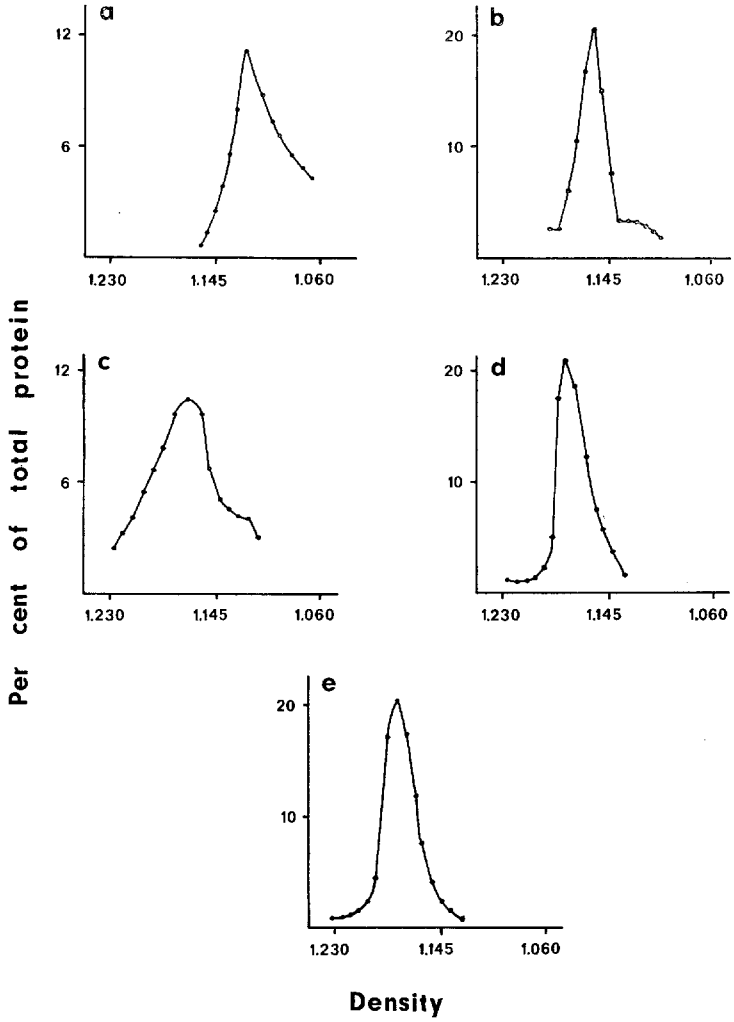

FIGURE 2 Density equilibration of smooth microsomal subfractions. After subfractionation, the gradient was collected in four fractions (1–4 from bottom to top) and the pellet was suspended in 0.25 M sucrose. 3 ml of the individual subfraction was layered on an appropriate continuous sucrose gradient and centrifuged for 16 hr at 50,000 *g*. *a*, fraction 4; *b*, fraction 3; *c*, fraction 2; *d*, fraction 1; *e*, pellet. Median equilibrium densities: *a*, 1.106; *b*, 1.145; *c*, 1.155; *d*, 1.176; *e*, 1.184.

parallel each other, are highly concentrated to the lower part of the gradient and decrease gradually towards the top. G6Pase exhibits an almost opposite pattern, being concentrated to membranes which sediment to the middle part of the gradient. IDPase is also high in the fractions which exhibit high G6Pase activity, and moreover shows an activity in the pellet almost equal to that of the total smooth microsomes. It is also apparent from Fig. 3 that correction of the specific activities for the sedimentable protein would not change the general pattern since all specific activities on the very top are low.

ELECTRON TRANSPORT ENZYMES: The activities or amounts of three components of the NADH-oxidase system (NADH → Fp → cyt.b_5 → (cyt. *c*))[3] and three enzymes of the NADPH-

[3] The three enzymes are: NADH-ferricyanide reductase, where the flavoprotein directly reduces the acceptor; cytochrome b_5; and NADH-cytochrome *c* reductase, which involves at least two components, Fp and cyt. b_5.

TABLE V

Protein, RNA, PLP, and Cholesterol Content of Smooth Microsomal Subfractions

After subfractionation of smooth microsomes, five fractions were collected by inserting a needle into the bottom of the tubes. Fraction 1 represents that part of the gradient which was located just above the pellet. The particulate components were pelleted at 105,000 *g* for 90 min. The pellets were suspended in distilled H_2O and used for chemical analysis. The values are the means of four experiments ± SEM.

Fraction	Protein	RNA*	PLP	Cholesterol	Cholesterol / PLP
			mg/fraction		
Total smooth microsomes	17.2 ± 2.1	1.14	6.2 ± 0.7	0.74 ± 0.07	0.12
Pellet	3.4 ± 0.6	0.14	1.30 ± 0.14	0.24 ± 0.04	0.185
Fraction 1	0.79 ± 0.1	0.039	0.30 ± 0.05	0.045 ± 0.006	0.15
2	1.33 ± 0.3	0.064	0.52 ± 0.06	0.056 ± 0.007	0.108
3	2.73 ± 0.4	0.16	1.10 ± 0.15	0.107 ± 0.012	0.097
4	4.30 ± 0.5	0.52	1.60 ± 0.24	0.154 ± 0.012	0.097
5	2.10 ± 0.4	0.19	0.73 ± 0.14	0.070 ± 0.011	0.096

* The mean of two experiments.

TABLE VI

Relative Specific Activity and Recovery of Phosphatases and Electron Transport Enzymes after Subfractionation

Subfractionation was performed as described in Materials and Methods. The values are the means ± SEM of four experiments.

Enzyme	Relative specific activity or amount in smooth microsomes	Recovery on the gradient
	per mg protein	%
AMPase*	1.3 ± 0.2	90 ± 12
ATPase*	2.0 ± 0.2	88 ± 6
G6Pase*	5.4 ± 1.1	78 ± 10
IDPase*	15.0 ± 2.3	87 ± 6
NADPH-NT reductase‡	0.074 ± 0.013	98 ± 8
NADPH-cyt. *c* reductase‡	0.045 ± 0.07	102 ± 7
NADH-cyt. *c* reductase‡	0.65 ± 0.15	99 ± 15
Cytochrome b_5§	0.43 ± 0.06	89 ± 16
Cytochrome P-450§	0.50 ± 0.10	79 ± 18

* μmoles P_i/20 min.

‡ μmoles NADPH or NADH ox./min.

§ mμmoles.

linked electron transport sequence (NADPH → Fp → x → cyt. P-450 → hydroxylation)[4] were also determined in the subfractions and are presented in Fig. 4. One of these, NADPH-cyt. *c* red. activity, displays greater stability, while the NT-reductase as well as the NADH-cyt. *c* red. are somewhat more labile. In spite of this, all electron transport enzymes, like the phosphatases, exhibit reproducible distribution and good recovery on the gradient. The same is valid for the cytochromes b_5 and P-450. The two cytochromes could not be measured in single fractions, and so the spectrophotometric analysis was performed on collected fractions from three gradients (Fig. 4). All electron transport enzymes follow a similar distribution pattern, according to which the upper third of the gradient displays a significant concentration. In this way, the membranes which are enriched in electron transport enzymes also have, in general, relatively low phosphatase activities, which further contrast the distribution pattern of microsomal oxido-reductive enzymes.

[4] The enzymes measured are: NADPH-cytochrome *c* reductase, mediated only by the flavoprotein; NADPH-NT reductase, probably involving a component additional to the Fp; and cytochrome P-450.

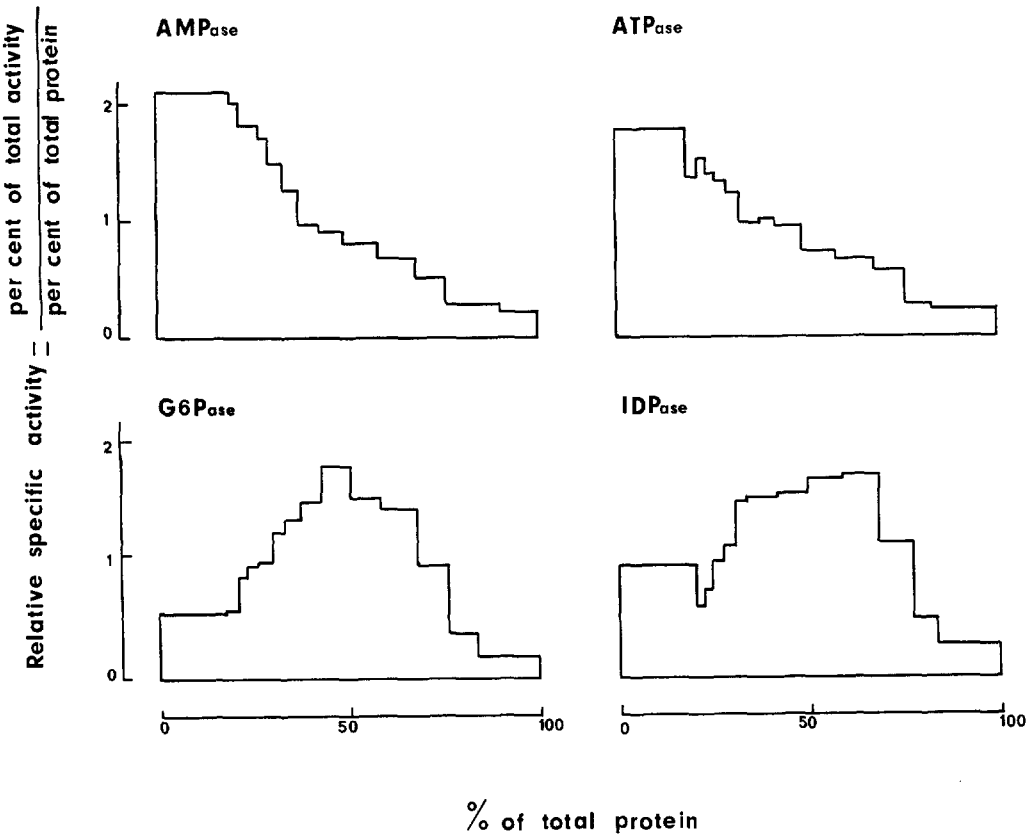

FIGURE 3 Distribution of phosphatases in smooth microsomal subfractions. The plot is made in the manner adopted by de Duve et al. (28). Relative protein content of fractions is shown on the abscissa, and relative specific activity on the ordinate. The column at the far left represents the pellet. The next corresponds to fraction 1 deriving from the lowest part of the gradient, since it was removed first by puncturing the bottom of the tube. In this way, the fractions at the right originate from the upper parts of the gradient. The values for the individual fractions represent the mean values of six experiments.

In order to elucidate whether the well established increase of NADPH-cyt. *c* red. activity after phenobarbital treatment is attributable to any specific membranes of smooth microsomes, subfractionation was performed after injection of phenobarbital (8 mg/100 g × 3 or × 5) (Fig. 5). There is an over-all enhancement in specific activity in all subfractions when compared to the control. There is a proportionally higher increase in fractions 5 and 6, but since the sedimentation velocity of the vesicles from phenobarbital-treated rat is not necessarily equal to that of the control, it would be premature to place too great a significance on this finding.

Incorporation Studies

Microsomal membrane components have relatively short half-lives, and some enrichment of newly synthesized lipids in one subfraction would give rise to an increase of radioactivity in labeling experiment (10, 23). Total PLP were labeled with glycerol-^{3}H and cholesterol with mevalonate-^{3}H, and the appropriate lipids were prepared during the linear phase of incorporation. Under such conditions, there is a similar labeling of all subfractions with either glycerol-^{3}H or mevalonate-^{3}H (Fig. 6). These experiments indicate that no concentration of newly synthesized membrane pieces is present in any of the isolated subfractions.

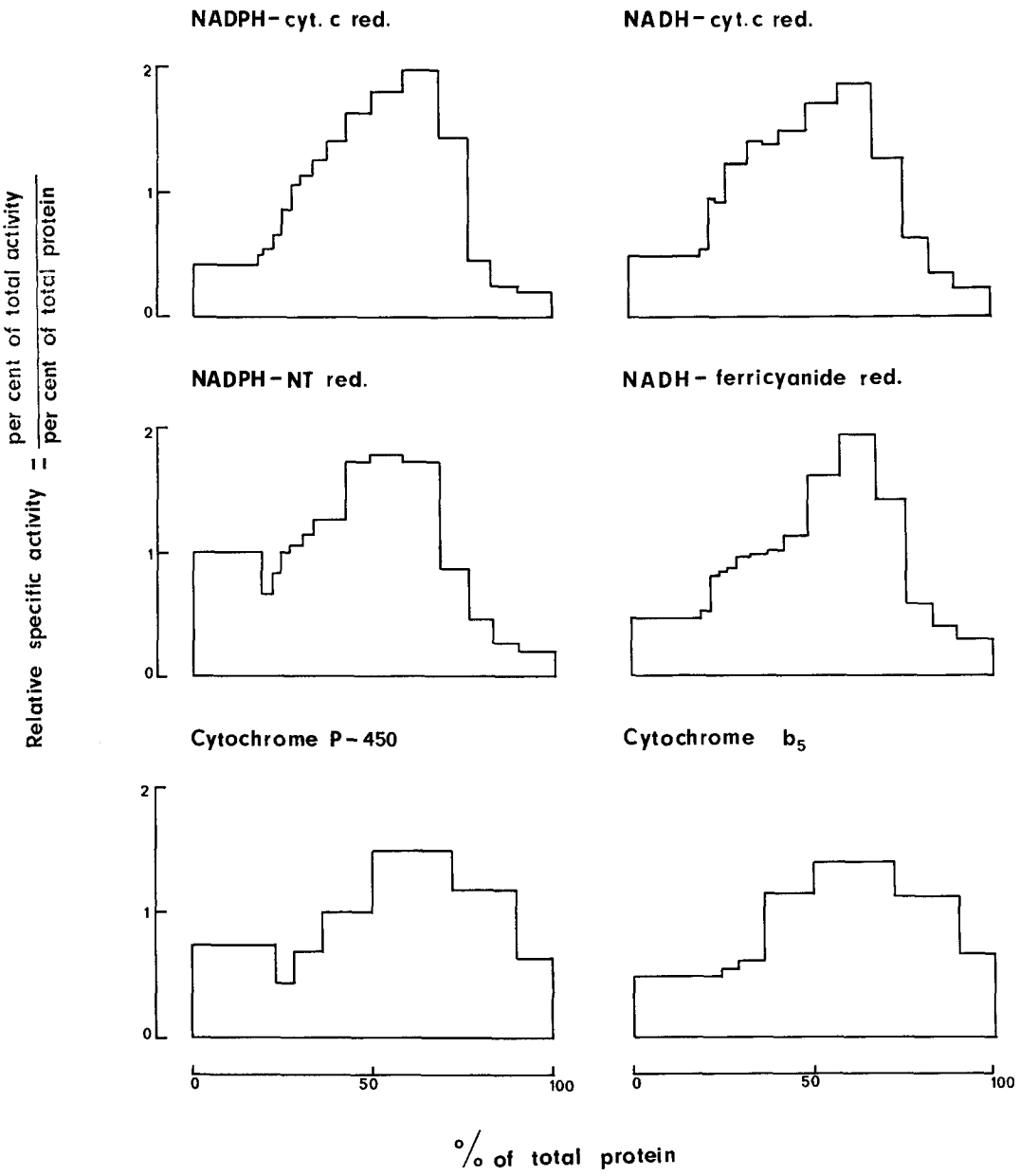

FIGURE 4 Distribution of electron transport enzymes in smooth microsomal subfractions. Distribution as presented in Fig. 3.

Contamination with Other Cytoplasmic Membranes

Since the definition of the microsomal fraction is operational (3), it is possible that other subcellular organelles, or particles from these organelles, sediment together with membranes of the ER. Using marker enzymes such as cytochrome *c* oxidase for mitochondria and acid phosphatase for lysosomes, it is apparent from Table VII that no appreciable contamination from these organelles occurs in any microsomal fraction. The

amount of MAOase activity in mitochondria is about five times higher than in the three microsomal subfractions together. On a protein basis, outer mitochondrial membranes exhibit a high concentration, a finding in agreement with previous data in the literature (32). The relatively high content of MAOase in both rough and smooth microsomes makes it reasonable to suppose that this enzyme exhibit as multimodal distribution (33).

AMPase activity is regarded by many authors as a good marker for plasma membranes (30, 34), but the possible occurrence of this enzyme in microsomes is also discussed (35). It is also well established that cholesterol is present in PLM (30, 34, 36), and the possibility of its absence from other subcellular fractions has been suggested (37). The total microsomal fraction subfractionated by gradient centrifugation in the presence of digitonin shows an enrichment of cholesterol in the low density membranes, which are also rich in AMPase activity (38). According to our previous investigations, microsomal subfractions contain cholesterol, but less than PLM (5, 6). When cell membranes of the liver cell are isolated on a discontinuous sucrose gradient, the fraction contains ∾ 0.15 mg cholesterol per gram of liver, which is only 5% of the amount present in the homogenate from perfused liver (Table VIII). If all the cholesterol of the hepatocyte is in PLM, this 5% would mean that the PLM recovery by this procedure is only 5% since cholesterol of the liver cell is almost exclusively particle bound. The enzymic data, such as AMPase distribution, do not support this interpretation. Furthermore, such a recovery would also lead to a total plasma membrane content of ∾ 34 mg protein per gram of liver, which is ∾ 15% of the total protein content in the homogenate. This amount is more than the protein of the mitochondrial or microsomal fractions, organelles which are much more abundant in electron micrographs. This again supports a true microsomal localization of cholesterol.

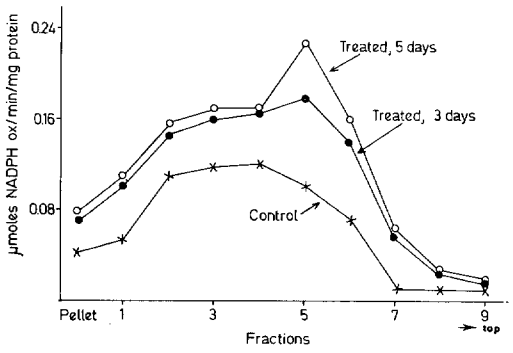

FIGURE 5 Comparison of NADPH-cytochrome *c* reductase activity in smooth microsomal subfractions of control and phenobarbital-treated rats (3 days and 5 days). Phenobarbital was given 8 mg/100 g once daily.

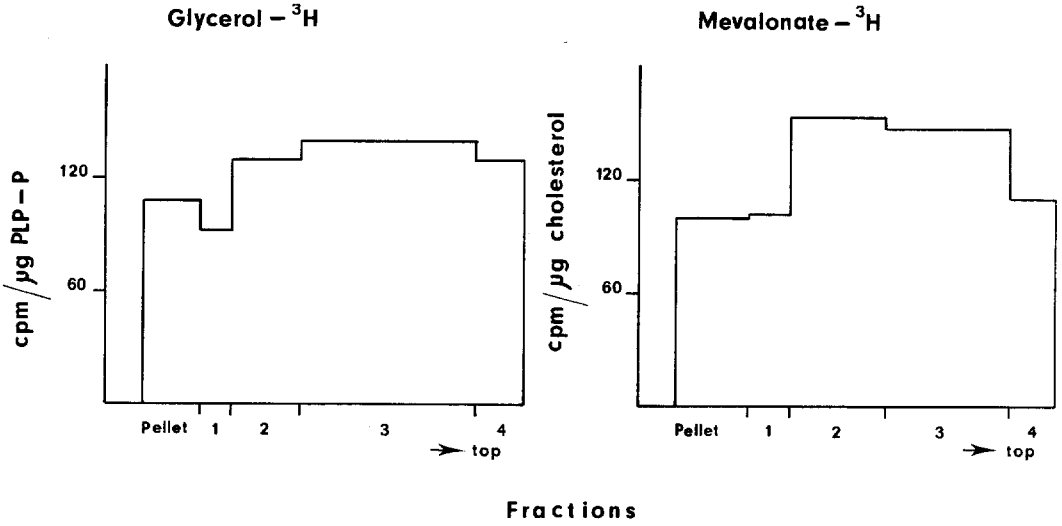

FIGURE 6 In vivo incorporation of glycerol-^{3}H into PLP (left), and mevalonate-^{3}H into cholesterol (right) of smooth microsomal subfractions. The incorporation time was 10 min in both cases.

TABLE VII

Distribution of Some Marker Enzymes in Different Subcellular Organelles

Plasma membranes were isolated by the method of Coleman et al. (30). Mitochondria and outer mitochondrial membranes were prepared according to Sottocasa et al. (31).

Fraction	MAOase*		Cyt. *c* oxidase‡	Acid Pase§
	per g of liver	*per mg protein*		
Homogenate				51.0
Plasma membranes	1.5	1.0		
Mitochondria	324	14.0	94.5	
Outer mitochondrial membranes	208	104		
Rough microsomes	35	3.1	0.5	3.0
Smooth I microsomes	19	2.8	0.3	2.9
Smooth II microsomes	3	2.0	0.2	0.6

* mmoles benzylamine/min.

‡ μmoles O_2/min/g of liver.

§ μmoles P_i/20 min/g of liver.

TABLE VIII

Comparison of Distribution of Protein, PLP, Cholesterol, and AMPase between Plasma Membranes and Microsomes

The fractions were prepared after liver perfusion with 0.25 M sucrose (vena portae). Isolation of PLM was performed according to (30).

	Protein	PLP	Cholesterol	Cholesterol PLP	AMPase	
					μmoles P_i/20 min	
	mg per g of liver				*per mg protein*	*per mg cholesterol*
Homogenate	228	20.0	3.0	0.15	0.62	47
Plasma membranes	1.7	0.51	0.15	0.29	12.7	144
Total microsomes	25	5.5	0.73	0.13	0.78	27
Rough microsomes	12.1	2.67	0.28	0.10	0.62	27
Smooth I microsomes	6.3	1.36	0.19	0.14	0.86	28
Smooth II microsomes	1.2	0.44	0.09	0.20	2.0	27

The data is Table VIII also show that 1.7 mg of plasma membrane protein is associated with 0.15 mg of cholesterol. If cholesterol were a marker of plasma membrane contamination, the smooth I fraction would consist of 35% and the smooth II fraction of 100% of plasma membrane elements. There are several experimental data proving the nonplasma membrane origin of the smooth II microsomes: this fraction contains high amounts of cytochromes b_5 and P-450, has relatively low AMPase and ATPase activities, participates in albumin transport, and can also be differentiated by its sphingomyeline content (6, 9, 29).

The exclusive plasma membrane localization of both AMPase and cholesterol are, furthermore, in disagreement with the data of Table VIII, since the AMPase per cholesterol ratio in the various microsomal subfractions is five times lower in comparison with plasma membranes.

Relationship to Previous Subfractionation Procedures

A series of smooth microsomal subfractions were prepared by Rothschild using isopycnic equilibrium centrifugation (14). The densities of the subfractions were in the same range as for the subfractions in our procedure (see Fig. 2). Also, smooth microsomes can be divided into two subfractions in the presence of Mg^{++}, one of which, smooth II, exhibits certain similarities to the

pellet in these experiments. In order to avoid the aggregating effect of Mg^{++} (see Tables I and II), relative enrichment of smooth II membranes was obtained by introducing an intermediary layer of 0.9 M sucrose between the heavy sucrose and the mitochondrial supernate during the separation procedure. The two smooth fractions, recovered at the two interphases, were subfractionated on the same gradient as used for all the other experiments. In both cases, a similar pattern of protein distribution to that of Table V was obtained. Also, the enzymic data in Fig. 4 disprove the hypothesis of there being a specific concentration of "smooth II type" membranes in the pellet.

DISCUSSION

The data presented in this paper point out the appreciable heterogeneity of the smooth microsomes in rat liver as regards both chemical and enzymic composition. The heterogeneity described appears to be an intrinsic property of the endoplasmic membrane system and not a result of various types of contamination artificially introduced by the subfractionation procedure.

There are a number of conditions which must be fulfilled for a successful subfractionation of smooth microsomes. The first is the requirement of a mild and short homogenization in order to avoid breakage of the plasma membranes as well as detachment of a part of the bound ribosomes. Spontaneous aggregation is not generally encountered in fractionation procedures of subcellular particles, but this is a serious problem in the case of smooth microsomes. Aggregation occurs often and under conditions generally used in fractionations, and the prevention of this undesirable phenomenon requires careful control. From this point of view, it is necessary to keep the protein and sucrose concentration above a critical limit and also to avoid package of the vesicles, especially sedimentation to the bottom of the centrifuge tube. Dilution of the microsomal suspension is deleterious not only for the integrity of the microsomes but it also interferes with enzyme stability. Taking these facts into consideration, high recoveries of smooth microsomal enzymes can be obtained even after a relatively long subfractionation procedure.

Enzymically, three types of membranes appear to be present in the smooth microsomal fraction, which are characterized by peak activities of electron transport enzymes, G6Pase and Mg^{++}-ATPase. Enzymic heterogeneity in total liver microsomes revealed by density gradient centrifugation is described by Amar-Costesec et al. (37). It is hardly questionable, on the basis of the data presented in this paper, that smooth membranes exhibit an enzymic specialization. The size of the membrane entity which is enzymically delimited to a restricted function cannot be given with certainty. It is quite reasonable to suppose that relatively large membrane pieces collect enzymes devoted to a restricted function, since the specific activities of the peak fractions are high. The presence of a certain amount of other enzymes in the same subfraction can be explained by cross-contamination or by the fact that a whole vesicle represents a larger membrane piece than the functionally specific "subunit".

Subfractionation of intracellular membranes is a useful tool in studies of biogenetic processes (39). It would be of value to be able to separate smooth endoplasmic membranes synthesized at various time points. If new ER membranes are synthesized within the rough-surfaced ER and subsequently transferred to the smooth compartment, the latter could be divided into "new" and "old" ones. Distribution of the cholesterol, the incorporation rate of glycerol-^{3}H into PLP, the phenobarbital effect, and the enzyme distribution do not lend support to the idea of an enrichment of new membranes in any of the subfractions isolated. Induction studies and the even turnover of membrane components indicate the simultaneous presence of old and new ER membranes. There can be several reasons for the lack of isolation of such units in the above subfractionation procedure. The separation is based on physico-chemical properties which do not necessarily distinguish between old and new membranes. Newly synthesized units may also be relatively small, and a broad distribution would prevent a separation of them. A further alternative is that the individual components exhibit a high rate of turnover, resulting in a short time-elapse between old and new pieces.

The heterogeneity of smooth microsomes also poses another problem. Estimation of microsomal contamination in separation procedures of cytoplasmic particles and organelles is generally based on the presence of marker enzymes. Such an approach, however, is not necessarily reliable, since a random distribution of various types of smooth vesicles in an isolated fraction or pellet cannot be expected to occur.

The reason behind the differential sedimentation during gradient centrifugation is an important question from the point of view of both separation and study of membrane properties. Since the subfractionation is performed on a stabilizing gradient in a zone centrifugation procedure, the two main factors deciding the sedimentation velocity are the size and the density of the vesicles (22, 40, 41). The density distribution, which is documented in Fig. 2, is in line with the sedimentation pattern obtained. On the other hand, the density values are far from being sufficient explanation for the observed differences in sedimentation velocity, and variation in size is certainly the deciding factor.

The size of microsomal vesicles, however, is not static (1, 4, 24). Because of the limited permeability of these membranes to sucrose, they behave as osmometers, and, depending on the osmotic activity of the medium, the size of the vesicle will vary. The various types of vesicles most probably exhibit different permeability properties, the exact determination of which is not possible at present. Such permeability differences are indicated by experiments in which smooth microsomes were subfractionated on a Ficoll gradient, a polymer not able to penetrate microsomal membranes. In this system, some of the ATPase-containing vesicles sediment slowly, while the others retain the sedimentation properties they exhibited in sucrose. The simplest explanation for this selective change in sedimentation velocity is a nonidentical permeability among vesicles. However, it must be borne in mind that the breakage of the tubular system at the moment of homogenization can well introduce size differences, since the vesicle size after gradient centrifugation is influenced to a great extent by the osmotic property of the individual vesicles.

The hypothetical differences in permeability can theoretically be caused by several factors: (*a*) The fractionation procedure may damage smooth vesicles randomly. Because of a well reproducible enzyme distribution pattern, this possibility is unlikely. (*b*) The fractionation procedure may change those smooth microsomes which, on the basis of pre-existing differences in structure and/or composition, are available for such changes. (*c*) Permeability differences among membranes could already exist in the intact cell either because of the physicochemical arrangement of membrane components (42, 43) or because of individualities in the chemical composition of the membranes (44, 45).

It is not yet possible to make a definite choice between (*b*) and (*c*), but the suggestion in (*c*) is more attractive. In order to decide between these alternatives, it will be necessary to study the physicochemical properties and chemical composition of the various types of membranes in great detail.

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