HETEROGENEOUS DISTRIBUTION OF ENZYMES IN SUBMICROSOMAL MEMBRANE FRAGMENTS

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ABSTRACT

Microsomal membranes are postulated to contain either a homogeneous arrangement of individual enzymes or groupings of functionally related enzymes. In the present study we attempt to distinguish between these hypotheses in subfractions of rough microsomes from rat liver. After sonication, the individual vesicles that make up the rough-membrane fraction average less than 1/100 of their previous mass. The vesicles in the sonicated suspension are fractionated roughly according to size on a continuous sucrose gradient. Enzyme activity or concentration in fractions of the gradient is expressed on a phospholipid basis. Fractions containing primarily small vesicles differ from those containing larger vesicles in a manner suggesting a certain degree of separation of NADH-linked from NADPH-linked enzymes. NADH-ferricyanide reductase, NADH-cytochrome *c* reductase and cytochrome b_5 are most concentrated within the large vesicles in the lowest third of the gradient. In contrast, NADPH-cytochrome c reductase and cytochrome P-450 are found in highest concentration in the small vesicles that make up the upper third of the gradient. The results suggest a nonrandom distribution of these two enzyme groups in the membranes of the endoplasmic reticulum.

INTRODUCTION

The rough-surfaced ER[1] of the hepatocyte, a tubular and cisternal membrane system with attached ribosomes, is the major site of production of cellular and secretory proteins (1, 2). Homogenization of the liver is thought to result in breakage of this tubular system with subsequent fusion of the free membrane edges to form vesicles of various size. These rough-surfaced microsomal membranes can be isolated from homogenates on a discontinuous sucrose gradient containing cesium ions, and appear to constitute a uniform subcellular fraction in respect to ultrastructural appearance and chemical composition (3).

Evidence of regional organization within the superficially uniform fraction of rough-surfaced membranes is scant. The ribosomes exhibit heterogeneity in that only certain of them, which are attached more firmly to the membrane, appear to be engaged in protein synthesis at any given time

[1] Abbreviations used are: ER, endoplasmic reticulum; DOC, deoxycholate; PLP, phospholipid; RNA, ribonucleic acid; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide-phosphate.

(4–6). In addition the distribution of ribosomes on the membrane surface does not seem uniform. The close groupings of ribosomes in the hepatocyte from a newborn rat become spaced farther apart during the first few days of life, leaving intervening smooth-surfaced zones (7). The membranous portions of the fraction also show some evidence of heterogeneity in respect to enzyme activity after fractionation of vesicles on a continuous sucrose gradient (8).

Two other approaches to the detection of heterogeneity within membranes have been (*a*) the use of detergents or enzymic break-up of the membrane and (*b*) mechanical disruption. The first method results in a fractional release of certain enzyme components of the membrane, but this release may be governed more by the binding properties of these molecules than by their regional distribution within the membrane (9–11). Furthermore, the solubilized components of membranes may be redistributed in a manner that bears no relationships to the native state (12). Sonication, on the other hand, appears to constitute a further application of the mechanical forces that result in the break-up of the ER during homogenization. This method has been used successfully in the preparation of various types of submitochondrial particles (13, 14) and, more recently, in the isolation of outer mitochondrial membranes (15). If fragments are small enough to contain a nonrandom distribution of protein molecules and if they also differ in physical properties, then it may be possible to separate them by various methods of centrifugation. The sonic disruption of membranes is, however, complicated by heat production and the concomitant danger of enzyme denaturation.

In the present study, sonication is utilized to produce submicrosomal vesicles with an estimated average volume of about $\frac{1}{100}$ that in unsonicated material. These vesicles are fractionated roughly according to size on a continuous sucrose gradient. Morphology, chemical composition, enzyme distribution, and rates of protein and phospholipid synthesis within these fractions are studied with the aim of distinguishing among some of the hypotheses that have been proposed for the structural make-up of microsomal membranes. The results indicate a partial separation of the NADH-linked enzymes from the NADPH-linked group. Some of this work has appeared in abstract form (16).

MATERIALS AND METHODS

Animals

Adult male albino rats weighing 180–250 g were used. The adult animals were starved for 16–20 hr before sacrifice. 3-day-old rats were starved for 12 hr by separating them from the mother. During this period of starvation the cage was supplied with warm (~35°C) and humid (~90% relative humidity) air. Where indicated, phenobarbital, dissolved in sterile Ringer's solution, was given intraperitoneally (8 mg/100 g) once daily.

Fractionation

Microsomes were subfractionated as described previously (3), with certain modifications. Livers were homogenized in 0.25 M sucrose at a tissue concentration of 20% (w/v) in the case of adults and 15% in the case of newborns. This homogenate was centrifuged at 10,000 *g* for 20 min. Sufficient 1 M CsCl was added to the supernatant to give a final concentration of 15 mM. 4.5 ml of this supernatant was layered over 2.0 ml of 1.30 M sucrose containing 15 mM CsCl. After 90-min centrifugation at 102,000 *g* (Spinco ultracentrifuge, 40.2 rotor, tube angle 40°; Spinco Div., Beckman Instruments, Inc., Fullerton, Calif.) the clear, reddish upper phase, the double layer at the gradient boundary (*smooth microsomes*), and most of the heavy sucrose lower phase were removed with a pipette provided with a rubber aspirator. The pellet and the fluffy layer appearing above it constituted the *total rough microsomes*. Pellets and fluffy layers from two centrifuge tubes were combined and suspended in 11 ml of water and centrifuged at 105,000 *g* for 60 min. The clear supernatant was discarded and the pellet was suspended either in water or in 0.2 M sucrose to yield rough microsomes from 1 g of liver in 1 ml. No significant difference in enzyme stability with sonication or storage was detected between these two suspending media. All but the preliminary experiments employed water.

Sonication

3-ml aliquots of rough microsomes were sonicated with the fine tip of a Branson sonifier (Branson Instruments, Inc., Stamford, Conn.) at a setting of 2.5 amp for ten 15-sec periods. Intervals of 1.5–2 min were allowed for cooling in a salt-ice bath to bring the temperature from about 8–10° after each sonication down to 2–4°. This degree of cooling was essential for good recovery of enzyme activity. Cooling in an ordinary ice bath brought the temperature of the sample to 22° after a 15-sec period of sonication; briefer cooling intervals resulted in a stepwise rise in temperature with each sonication.

Subfractionation of Sonicated Rough Microsomes

2.0–2.5 ml of a suspension of sonicated rough microsomes from 1 g liver/ml was layered over a continuous sucrose gradient (17) ranging from 0.29 M to 1.32 M. This amount approaches the maximum that can be fractionated on this gradient. Centrifugation was performed in an SW 25 rotor (Spinco, model L centrifuge) at 58,500 *g* for 16 hr. Fractions were collected through a hypodermic needle inserted near the bottom of the tube at the upper edge of the pellet. The pellet was resuspended in 4 ml of water.

The entire fractionation procedure is schematically illustrated in Fig. 1.

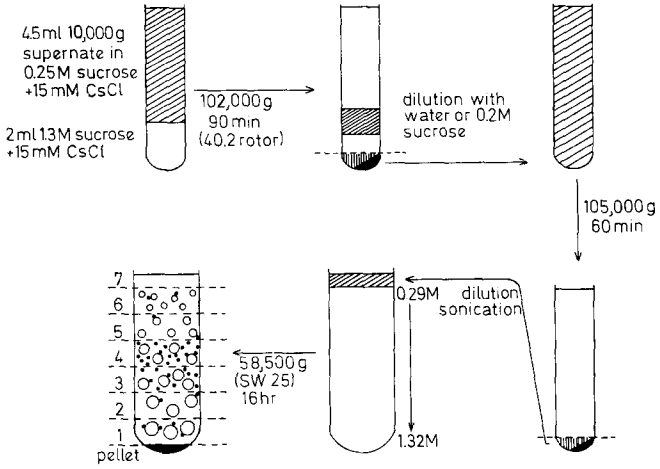


FIGURE 1 Schematic representation of the procedure for the isolation of rough microsomes and the subsequent subfractionation of sonicated membrane fragments.

Enzyme Assays

The activities of NADH-ferricyanide, NADH- and NADPH-cytochrome *c* reductase, lipid peroxidase, and glucose-6-phosphatase were determined as previously described (3, 18). Cytochromes b_5 and P-450 were determined spectrophotometrically as previously (3). The use of the Phoenix split beam spectrophotometer (Phoenix Precision Instrument Company, Philadelphia, Pa.) with a base line compensator resulted in improved accuracy of these determinations. NADH was used to reduce cytochrome b_5 (19) to eliminate the error due to hemoglobin contamination which may arise with $Na_2S_2O_4$ reduction. Cytochrome P-450, in addition to being measured after reduction with $Na_2S_2O_4$, was also determined after reduction with NADPH (20). Bubbling nitrogen through the samples before reduction resulted in identical spectra. Because interest was focused on the distribution of specific enzymes in relation to the membrane, enzyme activities were related to phospholipid rather than to protein content.

Incorporation Experiments

DL-leucine-1-^{14}C (20 mc/mmole) from The Radiochemical Centre, Amersham, England, was diluted in sterile Ringer's solution. 10 μc/100 g was injected intraperitoneally, and the rat was decapitated 90 min later. After pulse labeling, most of the radioactivity associated with albumin has disappeared from the liver after 60 min (21). At 90 min, therefore, contamination by nonmembranous protein should be minimized. Subfractionation of rough microsomes was carried out in the usual manner. Fractions from two sucrose gradients were combined and diluted with distilled water to a final volume of 30 ml, containing also 10 mM $MgCl_2$ and 0.15 M tris buffer, pH 8. Mg^{++} was utilized to aggregate small fragments to increase their sedimentation velocity (22), and the alkaline buffer was employed to release a portion of the adsorbed protein (23). Particulate matter was sedimented by centrifugation for 16 hr at 105,000 *g*.

The pellets were suspended in 0.53% NaDOC which solubilizes the membranous portion of the fraction and releases the ribosomes (1). Ribosomes were pelleted by 3 hr of centrifugation at 105,000 *g* and discarded. The supernatants were then dialyzed overnight against running tap water to reduce the DOC content. Protein was precipitated in 12% trichloroacetic acid and extracted to remove RNA and PLP by the method of Siekevitz (24). The protein precipitate was then dissolved in 0.9 ml of formic acid. Radioactivity was measured in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) with Bray's solution (25) as a scintillator. For protein determination by the method of Lowry et al. (26), one part of the formic acid solution was neutralized with 14 parts of 2 N NaOH.

Glycerol-2-^{3}H (50 mc/mmole) from The Radiochemical Centre was diluted in sterile Ringer's solu-

tion. 40 μc/100 g was injected intraperitoneally. The animals were decapitated after 10 min, and subfractions of rough microsomes were prepared from two gradients. Incorporation of glycerol-^{3}H into PLP is linear up to 25 min (23). PLP was extracted as described above and separated from neutral lipids on a silicic acid column by the method of Borgström (27). Although triglycerides make up only about 6% of the extracted lipids, they are rapidly labeled and constitute an important source of radioactive contamination (23). Radioactivity in the PLP extracts was measured with a toluene scintillator (28).

Free ribosomes, labeled in vivo, were obtained as follows: 300 μc of orotic acid-5-^{3}H (7 c/mmole, Radiochemical Centre) was injected intraperitoneally into a 200 g rat. After 24 hr the animal was decapitated. Total microsomes were prepared by centrifuging the mitochondrial supernatant for 60 min at 105,000 g. NaDOC was added to a final concentration of 0.53% to release the ribosomes. The remaining primarily nonribosomal material was removed by centrifugation for 15 min at 15,000 g. Then the ribosomes in the supernatant were pelleted by centrifugation for 2 hr at 105,000 g. This pellet was suspended in 1 ml water/1.5 g original liver. After centrifugation, the subfractions were precipitated with 12% trichloroacetic acid washed twice with 5% trichloroacetic acid, and the RNA was extracted with 5% trichloroacetic acid at 90° for 20 min. Aliquots were taken for determination of RNA by the orcinol reaction (29) and were added to 10 ml of Bray's solution for measurement of radioactivity.

Chemical Analysis

Protein was determined according to Lowry et al. (26) with bovine serum albumin as standard. RNA and PLP were measured as described previously (7). For measurement of cholesterol the chloroform extract was passed through a silicic acid-containing column (27) to separate neutral lipids from PLP. Total cholesterol was determined by analysis of the chloroform phase (30).

Millipore Filtration

A rough estimate of microsomal size could be obtained with Millipore filters (22). 3 ml of the fraction was filtered through Millipore filters of known pore size (Millipore Filter Corp., Bedford, Massachusetts) with the aid of a suction pump. The PLP concentration of the filtrate was compared to that of the original suspension in order to estimate the amount of microsomal material which passed through the filter.

Electron Microscopy

After subfractionation of rough microsomes, the gradient was collected in three 10-ml fractions, an upper, a middle, and a lower one in addition to the pellet. Distilled water was then added dropwise with continuous stirring to the fractions to a volume of 30 ml. The pellet was fixed *in situ* or was suspended in 30 ml of 0.25 M sucrose. After centrifugation in an SW 25 rotor at 58,500 g for 8 hr (lower fraction and pellet) to 15 hr (upper and middle fractions), the supernatants were decanted and the pellets were fixed overnight at 4° *in situ* by adding 1% osmium tetroxide solution buffered at pH 7.2 with phosphate. Small blocks including both the upper and lower parts were cut from the pellets, dehydrated in graded solutions of acetone, and embedded in Vestopal W. Thin sections were cut on an LKB microtome and mounted on Formvar-coated copper grids. They were double stained with uranyl acetate followed by "alkaline lead" solution (31). Electron micrographs were made with a Siemens Elmiskop I.

RESULTS

In efforts to isolate cellular fractions or subfractions there is an inherent risk of interpreting the results of an unrecognized artifact as successful fractionation. For this reason the characteristics of starting material and the fractions are discussed in some detail in this and subsequent sections.

Sonication

In preliminary experiments the effects of sonication upon sedimentation of membrane particles and upon enzyme recovery were determined. The number of 15-sec periods of sonication was varied from 1 to 20 at the 2.5 amp setting of the Branson sonifier. The sonicated rough microsomal suspension was layered on sucrose gradients with a range from 0.15 to 0.59 M. An increasing number of sonications resulted in a greater proportion of protein and PLP near the top of the gradient and less in the pellet, indicating a progressive decrease in average fragment size. Beyond 10 sonications there was little change in distribution of material on the gradient but an increasing loss of activity of the NADH- and NADPH-cytochrome c reductases. 10 sonications were selected for subsequent studies as optimal.

Recovery of Enzymes

Table I summarizes the recovery of several microsomal enzymes after 10 sonications. The recoveries of NADH-cytochrome c reductase, NADH-ferricyanide reductase, and NADPH-cytochrome c reductase activities average about 90%. Recoveries of cytochrome b_5 and cytochrome

P-450 are less complete with averages of 69 and 80%, respectively.

Table II shows the effect of 24-hr storage at 4° at the extremes of sucrose concentration and pH encountered during the fractionation. The sucrose concentrations represent the range from 0.29 M in fraction 6 to 1.32 M in fraction 1 of the gradient. Although the membrane protein has buffering capacity, the pH can occasionally be as low as 6.2 in the fractions at the time they are collected. The use of exogenous buffers was avoided because ionic media increase the tendency of membrane vesicles to aggregate (22). Recoveries of the reductases and cytochrome b_5 are above 80% under all conditions. As much as half of the cytochrome P-450 is lost at low sucrose concentrations, whereas a high sucrose concentration at pH 7.5 seems to have a stabilizing effect. The stability of these enzymes in a suspension of rough membrane fraction from 1 g liver/ml water is identical to that in a 1:10 dilution of this suspension during 24 hr of storage at 4°. The results for smooth membranes are shown for comparison. The marked lability of cytochrome P-450 in this fraction is noteworthy.

Conditions of Centrifugation

A 16-hr period of centrifugation on a gradient ranging from 0.29 to 1.32 M sucrose was selected

TABLE I

Recovery of Enzymes after Sonication and Fractionation of Rough Microsomes

	Recovery after sonication		Recovery on gradient	
	Range	No. of Exp.	Range	No. of Exn.
	%		%	
NADH-cytochrome *c* reductase	71–103	7	83–123	7
NADH-ferricyanide reductase	69–112	6	97–138	6
NADPH-cytochrome *c* reductase	83–104	9	80–101	9
Cytochrome b_5	56–77	5	77–86	4
Cytochrome P-450	74–84	5	84, 100	2

TABLE II

Enzyme Recovery After 24-Hr Storage

During storage the concentration of all samples corresponded to rough or smooth microsomes from 100 mg liver/ml. Smooth membranes were isolated as described in Materials and Methods and pelleted by centrifugation at 105,000 *g* for 120 min. The pH was adjusted with phosphate buffer to a final concentration of 0.05 M. The samples were stored at 4°. Recovery is expressed as per cent of the activity or amount in the original sample.

	NADH-ferricyanide reductase	NADH-cyt. *c* reductase	NADPH-cyt. *c* reductase	Cytochrome b_5	Cytochrome P-450
Rough microsomes, 0.29 M sucrose, pH 7.5	86	—	81	94	44
Sonicated rough microsomes, 0.29 M sucrose, pH 7.5	91	113	85	79	51
Sonicated rough microsomes, 0.29 M sucrose, pH 6.0	84	99	94	92	53
Sonicated rough microsomes, 1.32 M sucrose, pH 7.5	82	99	99	92	83
Sonicated rough microsomes, 1.32 M sucrose, pH 6.0	84	99	90	96	62
Smooth microsomes, 0.29 M sucrose, pH 7.5	117	107	83	92	17

after preliminary experiments, varying gradient range and centrifugation time, as these conditions result in a relatively even distribution of PLP. RNA is concentrated near the middle of the gradient as shown on Fig. 2*a*. Fig. 2*b* shows that a 44-hr centrifugation on the same gradient results in a greater sedimentation of PLP and RNA, indicating that the particles are not at equilibrium at the end of the 16-hr centrifugation period. Because of the small size of the particle and the high viscosity of the medium, attainment of isopycnic equilibrium was unrealistic (32). After the 16-hr period of centrifugation separation of particles occurs on the basis of both size and density.

Electron Microscopic Appearance

Rough-surfaced vesicles after isolation on the cesium-containing sucrose gradient are shown in Fig. 3. Vesicles vary between 200 and 400 mμ in diameter and have abundant ribosomes attached to their outer surface.

Figs. 4–7 show the appearance of fractions of the gradient after pelleting and fixation in buffered osmium tetroxide. Fig. 4 corresponds to the top third of the gradient and shows small vesicles ranging in size from 30 to 50 mμ with rare attached ribosomes. Fig. 5 depicts the middle third of the gradient. This fraction shows abundant free ribosomes. Vesicles are somewhat larger than in the top third, and it is difficult to distinguish what proportion of the surrounding ribosomes are attached. The bottom third of the gradient (Fig. 6) contains vesicles ranging in size from 60 to 110 mμ with relatively few free or attached ribosomes. The pellet (Fig. 7) resembles the bottom third of the gradient in most respects. The vesicles, however, are somewhat irregular in shape, perhaps due to packing. The electron microscopic appearance of pellets fixed *in situ* is identical to that of pellets fixed after resuspension and recentrifugation in 0.25 M sucrose.

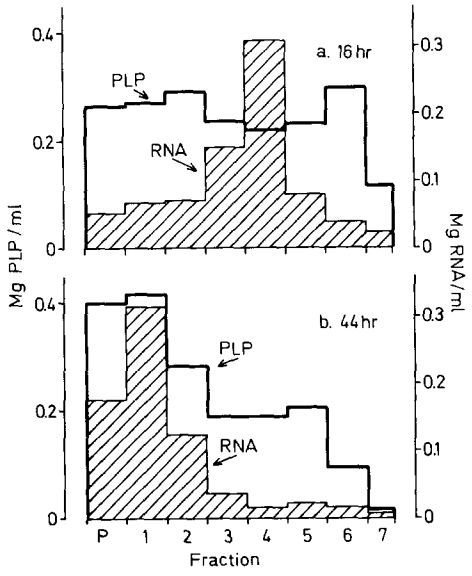


FIGURE 2 Distribution of PLP and RNA after prolonged centrifugation of sonicated rough microsomes. Sucrose gradients ranged from 0.29 to 1.32 M. Centrifugation time: (*a*) 16 hr and (*b*) 44 hr at 58,500 *g*. P represents the pellet, and the fractions are numbered from the lower part of the gradient toward the origin. The width of the column is proportional to the volume of the fractions. The pellet is suspended in 4 ml water.

In general, one can conclude that after sonication the average diameter of the sectioned vesicles is reduced. Since slices through vesicles may be tangential as well as through the larger diameters, the average diameter of membrane globules will be greater than apparent in the two-dimensional views. Since the volume of the vesicle shell is a function of the diameter raised to the third power, the average particle size is estimated to be reduced more than 100-fold by sonication. This conclusion is strengthened by the observation that sonicated vesicles are completely permeable to sucrose and, in contrast to unsonicated rough microsomal vesicles, do not show an osmotic response (33). For this reason the size of vesicles, upon electron microscopic examination, is probably less sensitive to the suspending medium and does not represent secondary changes introduced during the procedures employed. Ribosomes are apparently removed from the membranes to a large extent, but it is difficult to distinguish whether or not individual ribosomes are membrane bounded.

Millipore Filtration

In agreement with the morphological data, Millipore filtration experiments indicate a gradation of particle size along the gradient. Table III shows that fractions 5 and 6 pass readily through a filter of 50 mμ pore diameter, whereas fractions 1 and 2 are stopped by the 100 mμ pore size. Attempts to separate particles of different size, such as the ribosomes from the vesicles in fractions 3 and 4, were generally unsuccessful, probably because larger-sized particles plugged the pores.

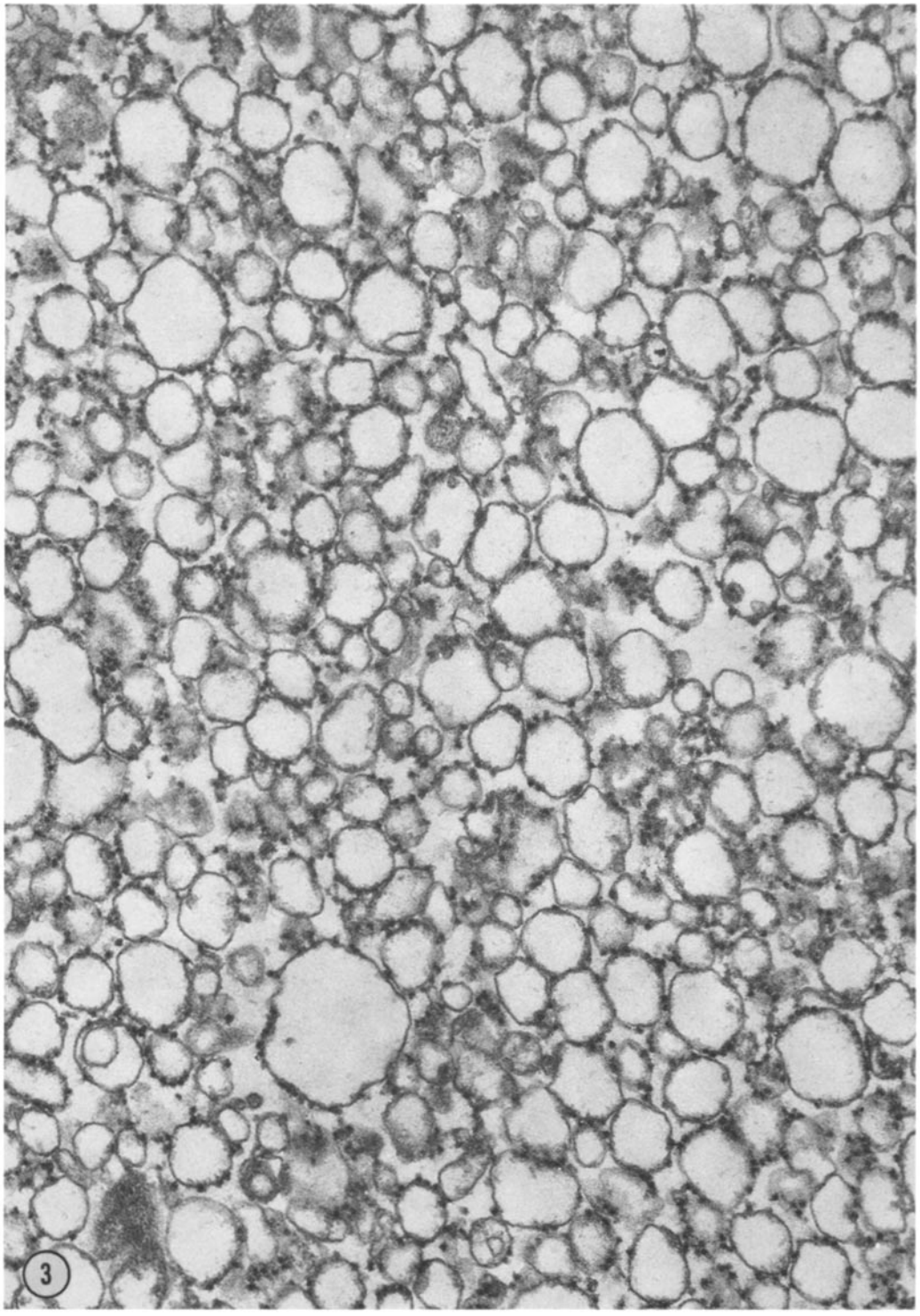
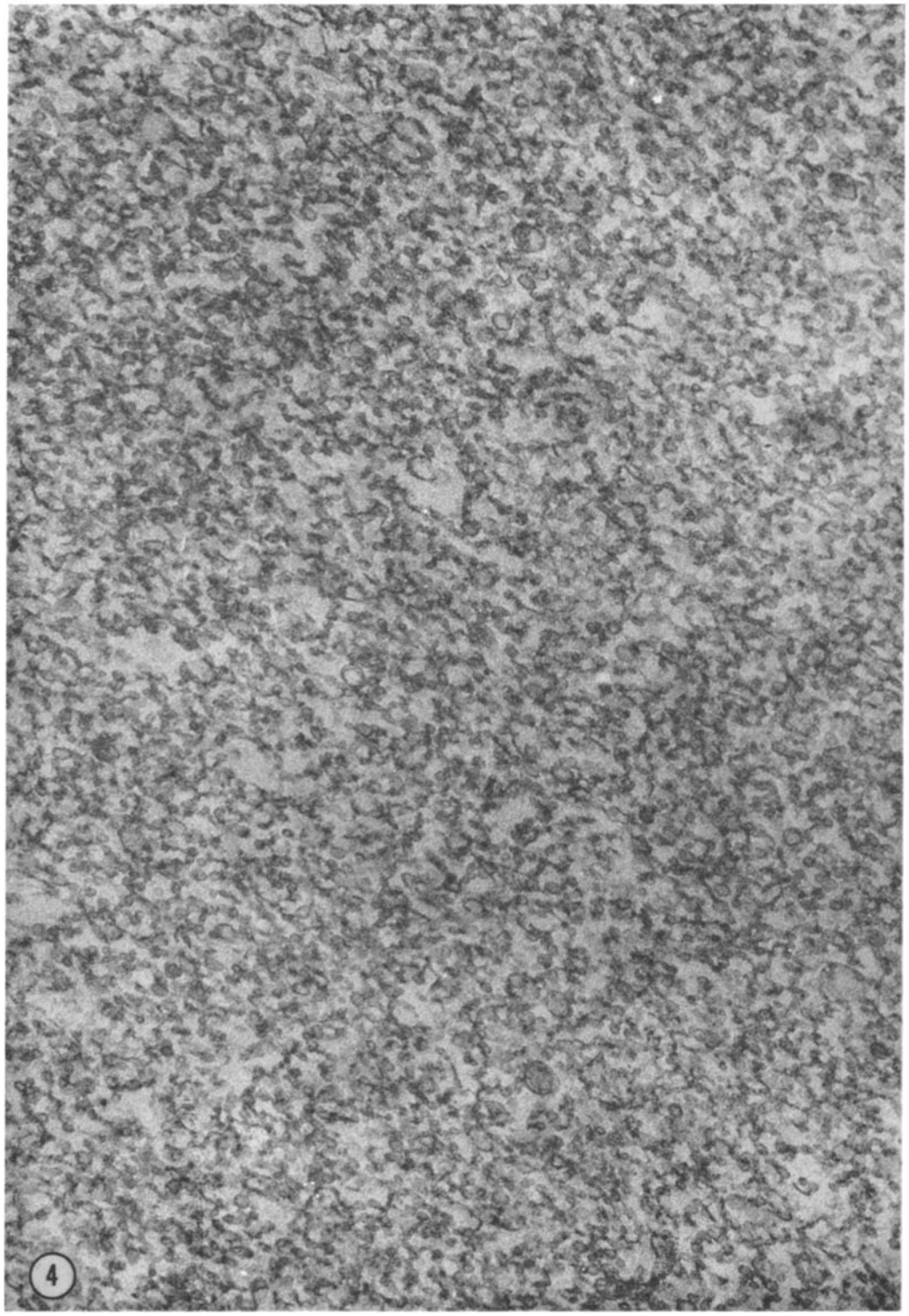


FIGURE 3 Isolated total rough microsomes before sonication. Vesicles with a diameter between 200 and 400 mμ. A large number of ribosomes are attached to the outer surface of all vesicles. × 60,000.



FIGURES 4–7 Appearance of subfractions of sonicated rough microsomes.
FIGURE 4 Resedimented microsomal material from the upper third of the gradient. Small vesicles ranging in size from 30 to 50 mμ. × 60,000.

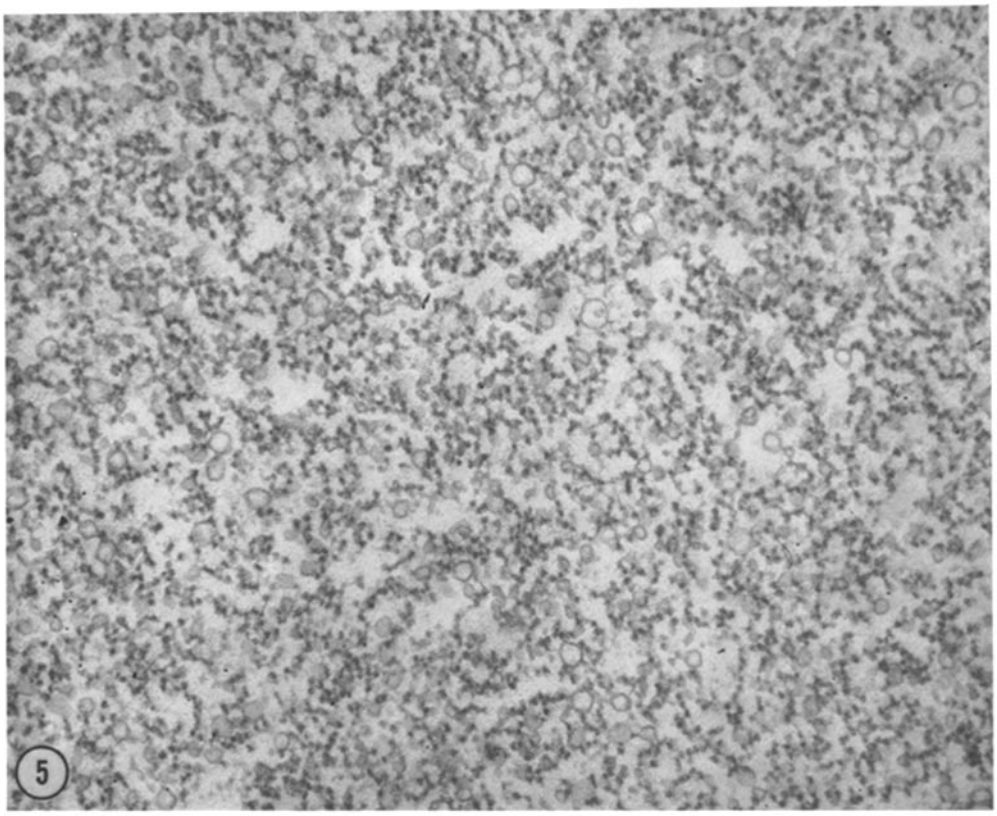


FIGURE 5 Resedimented subfraction from the middle third of the gradient. The field is covered by a large number of free ribosomes. Among these, vesicles can be discerned that are somewhat larger in size than in Fig. 4. × 60,000.

Chemical Composition

The chemical composition of total rough microsomes is shown in Table IV. Fig. 8 shows the distributions of PLP, protein, and RNA in individual fractions of the gradient. In samples from the adult there is a fairly even distribution of PLP on the gradient with two small peaks, one near the bottom of the tube in fraction 2 and the other near the top in fractions 5 and 6.

RNA is concentrated in fractions 3 and 4 in agreement with the morphological demonstration of large numbers of ribosomes in this middle third of the gradient (Fig. 5).

The distribution of protein on the gradient is quite even, apparently as a result of a major protein contribution by the ribosomes in the middle of the gradient, whereas the membrane contribution predominates in the upper and lower thirds. Fraction 7, which largely represents water in which the rough microsomes were layered onto the gradient, has a relatively high protein content but very little PLP or RNA. This fraction should concentrate nonparticulate components of rough microsomal fraction released by sonication. This released protein, probably mainly albumin, represents ~ 3% of the total protein applied to the gradient.

Increased amounts of ER are characteristic of both 3-day-old rats (7) and adults treated with phenobarbital (34). The distribution of PLP, RNA, and protein in the rough-surfaced membranes of these groups is similar to that of the untreated adult. The yield of total rough membrane PLP and protein in the newborn and the yield of RNA in the phenobarbital-treated animal are lower than in the untreated adult (Table IV and Fig. 8).

The content of cholesterol in smooth microsomal

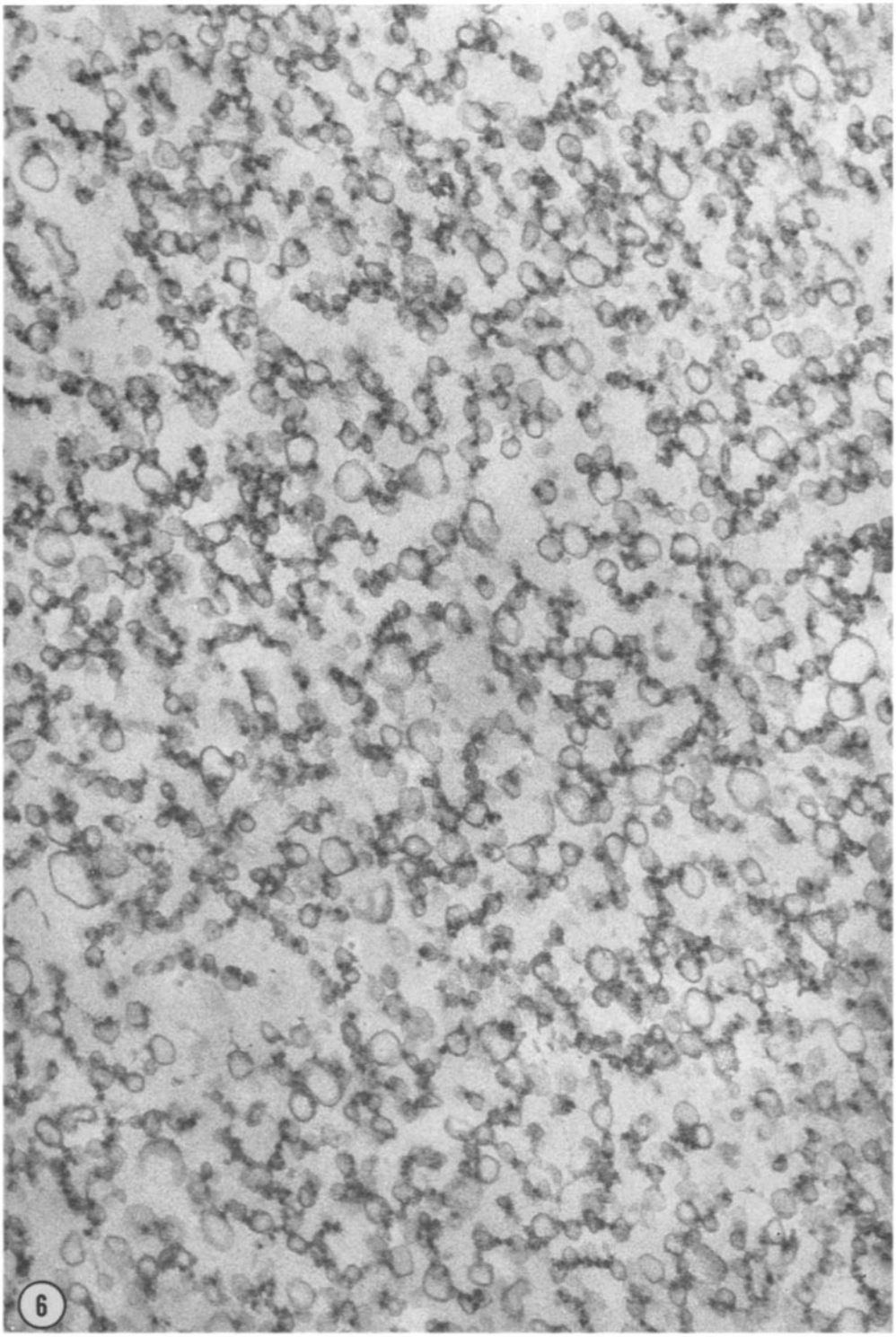


FIGURE 6 Repelleted microsomes from the lower third of the gradient. The average vesicle size is larger than in the previous two fractions, with an estimated range of 60–110 mμ in diameter. There are relatively few free or attached ribosomes. × 60,000.

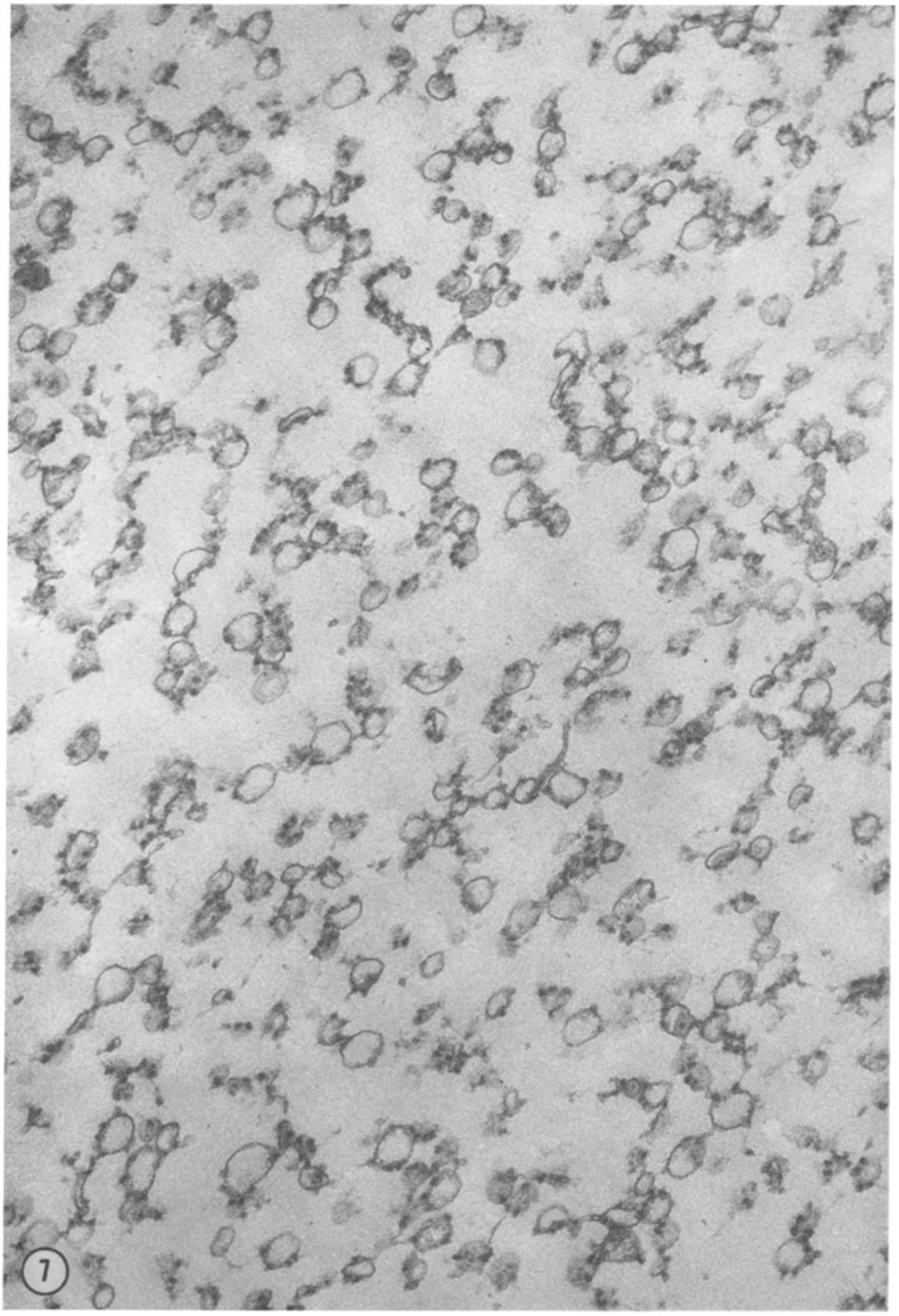


FIGURE 7 Pellet fixed *in situ* after subfractionation. The vesicle morphology is similar to that of Fig. 6. but the vesicles are somewhat more irregular in shape. Around the vesicles are a small number of ribosomes, a few of which are attached. × 60,000.

membranes is significantly higher than in rough membranes (23). It is generally agreed that smooth membranes develop from rough-surfaced segments (7). In this case it might be possible to isolate a fraction rich in cholesterol that would correspond to "smooth patches" within rough membranes. Table V shows that this was not observed. The ratio of cholesterol to PLP is quite uniform in various fractions. Only the pellet appears to have a somewhat lower cholesterol content.

In order to determine whether the bulk of the ribosomes removed by sonication is free or is still attached to fragments of membrane, labeled free ribosomes were prepared from a rat injected intraperitoneally with 300 μc of orotic acid-^{3}H 24 hr previously. Three gradients were layered as follows: the first with sonicated rough microsomes, the second with labeled free ribosomes, and the third with labeled free ribosomes and sonicated rough microsomes. As shown in Fig. 9 the peak of labeled free ribosomes corresponds closely to the RNA peak of the sonicated rough microsomes. These results indicate that sonication removes most ribosomes from their membrane attachments. Nevertheless, the possibility that small membrane fragments are attached to the ribosomes cannot be entirely excluded. Fig. 9 also indicates that free ribosomes sedimented more rapidly in the gradient that did not contain added rough membranes,

TABLE III

Passage of Fractions through Millipore Filters

Unsonicated and sonicated rough microsomes from 300 mg liver in 3 ml of 0.25 M sucrose or 3 ml of undiluted single or combined fractions were applied to the filter. Numbers denote the per cent of the original PLP concentration found in the filtrate. Dash means no or minimal passage through filter.

Sample	Millipore filters, pore size				
	450	300	220	100	50
Unsonicated rough microsomes	72	30	—	—	—
Sonicatted rough microsomes		103	94	—	—
Fractions 1 + 2			94	—	—
3 + 4			85	83	—
5			92	82	68
6			97	98	89

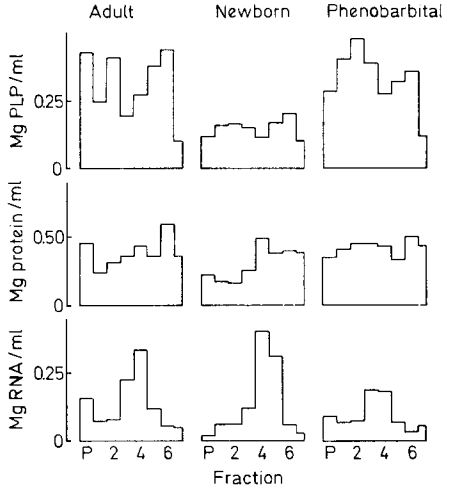


FIGURE 8 Distribution of PLP, protein, and RNA in subfractions obtained from adult, newborn, and phenobarbital-treated rats. The width of the bars is proportional to the volume of the fraction; the pellet is suspended in 4 ml water. Phenobarbital (8 mg/100 g per day × 3) was injected intraperitoneally.

TABLE IV

Chemical and Enzyme Composition of Rough Microsomes

Values are for rough microsomes obtained from 1 g liver in each of three representative experiments.

	PLP	Protein	RNA	NADH-ferricyanide reductase	NADH-cyt. *c* reductase	NAPDH-cyt. *c* reductase	Cyto-chrome b_5	Cyto-chrome P-450
	mg	*mg*	*mg*	*μmoles NADH oxidized/min*	*μmoles cytochrome c reduced/min*		*mμmoles*	
Adult	5.5	13.4	2.4	31.1	10.3	0.76	78.0	63.1
Newborn, 3-day-old	2.3	10.3	2.3	7.7	2.3	0.69	36.7	12.3
Adult, phenobarbital-treated (8 mg/100 g per day × 3)	6.0	14.1	1.6	29.0	7.0	3.08	110.1	105.0

probably because of an aggregation of rough membranes and free ribosomes.

Incorporation of Labeled Precursors into Protein and Phospholipid

The incorporation of leucine-^{14}C into isolated total membrane protein is relatively uniform along the entire gradient when adult, 3-day-old, and adult rats treated with phenobarbital for 3 and 6 days are investigated. The incorporation of glycerol-^{3}H into total PLP is also uniform. There is therefore no evidence for a newly synthesized or early labeled fraction of membrane vesicles.

Enzyme Distribution

The enzyme composition of total rough microsomes is shown in Table IV. In the 3-day-old rat the NADH-reductases and the two cytochromes are substantially below adult levels per g liver. In phenobarbital-treated rats the activity of NADPH-cytochrome *c* reductase and the concentration of cytochrome P-450 are greatly increased.

NADH- and NADPH-Cytochrome C Reductases, and NADH-Ferricyanide Reductase

Though the first reaction in this group requires more than one intermediate, the latter two reactions appear to involve only a single enzyme. Thus ferricyanide reductase is dependent only upon the flavoprotein, whereas NADH-cytochrome *c* reductase requires the participation of cytochrome b_5 as well (35, 36). Thus disruption of linkages within the chain or loss of essential cofactors might be more likely to decrease the activity of the latter enzyme reaction. The distributions of the two NADH-reductases are similar in most respects in any given experiment but differ considerably from that of NADPH-cytochrome *c* reductase. For the sake of clarity, only the cytochrome *c* reductases are shown in Figs. 10 *a* and 10 *b*. There is a moderate enrichment of NADH-cytochrome *c* reductase in the lower third of the

TABLE V

Cholesterol and PLP Content of Fractions

Fractions from three gradients were combined. The values for total rough microsomes represent the equivalent of 7.5 g liver or 2.5 g liver/gradient.

	Cholesterol	PLP	Cholesterol / PLP
	mg/sample	*mg/sample*	
Total rough microsomes	1.50	22.5	0.062
Pellet	0.146	3.15	0.046
Fraction 1	0.187	2.70	0.069
2	0.221	3.06	0.072
3	0.139	2.16	0.064
4	0.167	2.27	0.074
5	0.194	2.59	0.075
6	0.225	3.18	0.071

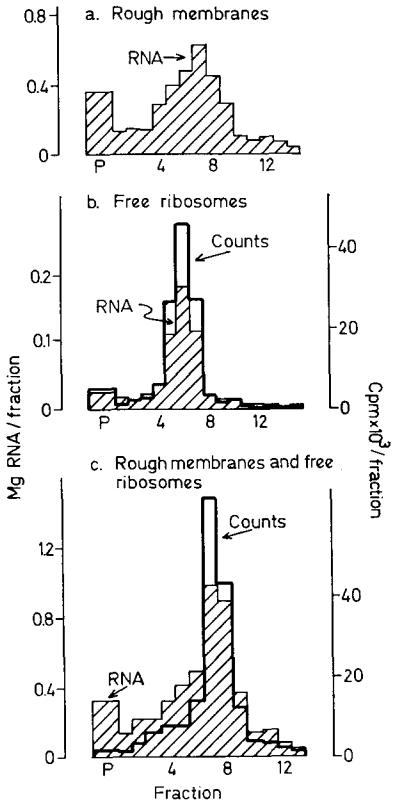


FIGURE 9 Sedimentation of free ribosomes and rough-surfaced membranes on the sucrose gradient. Sonicated rough microsomes from 2 g liver in 2.5 ml water are layered on gradient *a*. Free ribosomes labelled in vivo with orotic acid-^{3}H were prepared with 0.5% DOC. Free ribosomes from 0.75 g liver in 2.5 ml water are layered on gradient *b*. The same amount of sonicated rough microsomes as in *a* and free ribosomes as in *b* are combined in a total volume of 2.5 ml water and layered on gradient *c*. The fractions are approximately 2 ml and the pellet is suspended in 4 ml water.

gradient in relation to unfractionated material on PLP basis. In the upper part of the gradient there is a progressive decline of this enzyme activity. NADH-ferricyanide reductase activity in the top fractions of the gradient is decreased somewhat less than the activity of NADH-cytochrome *c* reductase in most experiments. This is illustrated in Table VI. Both the NADH-cytochrome *c* reductase/NADPH-cytochrome *c* reductase ratio and the NADH-ferricyanide reductase/NADPH-

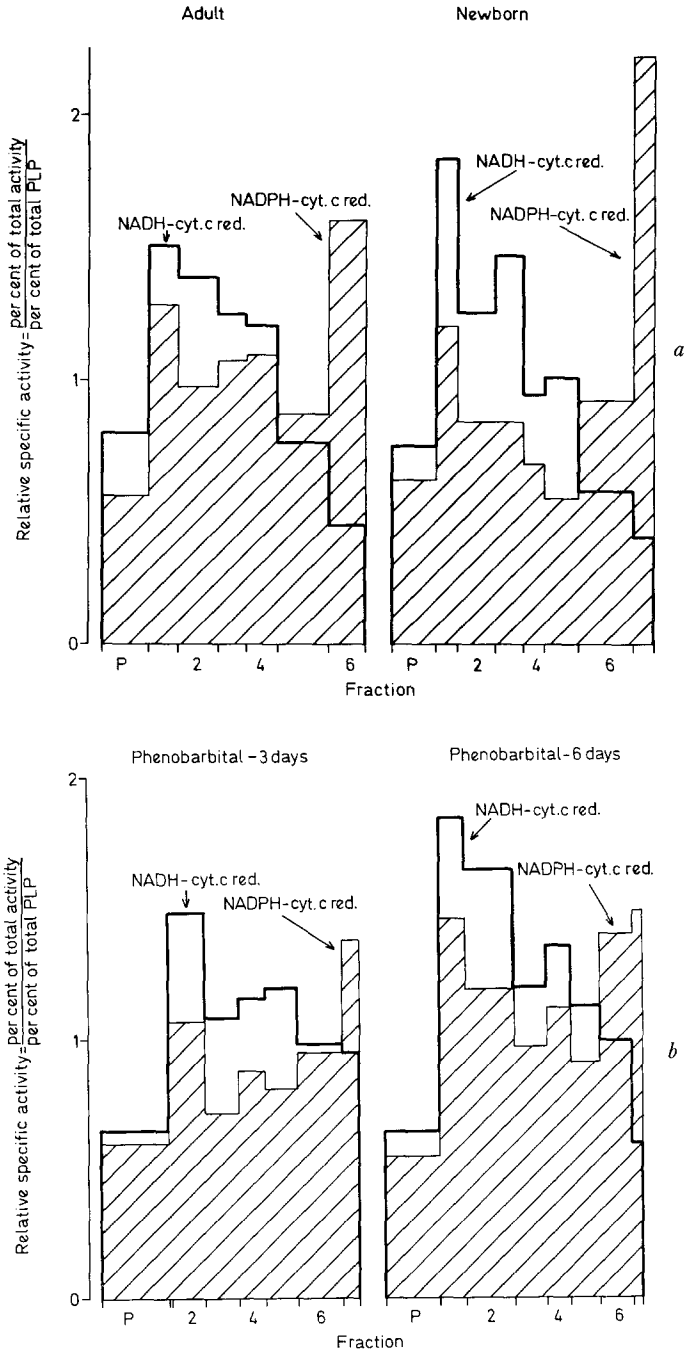


FIGURE 10 Distribution of NADH- and NADPH-cytochrome c reductase activity in subfractions. The plot is made in the manner adopted by de Duve et al. (37). Relative PLP content of fractions is shown on the abscissa and the relative specific activity on the ordinate. Fig. 10 *a* Distribution of enzyme activities in the adult (left) and newborn, 3-day-old (right). Fig. 10 *b* Distribution of enzyme activities in adult, treated with phenobarbital (8 mg/100 g per day) for 3 days (left) and 6 days (right).

TABLE VI

Enzyme Composition of Subfractions: Reductases and Lipid Peroxidation

Rough microsomes derived from 1 g liver were suspended in 1 ml water. Fractions were derived from a gradient layered with 2.5 ml rough microsomal suspension. The pellet was suspended in 4 ml water. Enzyme activities are expressed per ml of the unfractionated rough microsomes and of the individual fractions. Lipid peroxidase was determined in a separate experiment. Enzyme ratios are based upon a value of 1.00 in the sonicated rough microsomes.

	NADH-ferricyanide red.	NADH-cyt. *c* red.	NADPH-cyt. *c* red.	Lipid perox.	NADH-ferricyanide red. / NADPH-cyt. *c* red.	NADH-cyt. *c* red. / NADPH-cyt. *c* red.
	μmoles NADH oxidized/min per ml	*μmoles cyt. c red./min per ml*		*mμmoles O_2/min/ml*		
Rough microsomes	29.0	13.8	0.75	186	0.96	1.34
Rough microsomes, sonicated	31.1	10.3	0.75	192	1.00	1.00
Pellet	1.69	0.61	0.058	14.9	0.71	0.77
Fraction 1	2.18	0.77	0.054	17.9	0.97	1.04
2	2.34	0.91	0.055	33.2	1.70	1.21
3	1.50	0.54	0.043	22.2	0.84	0.92
4	1.66	0.64	0.040	15.6	1.00	1.17
5	2.10	0.63	0.057	15.6	0.89	0.80
6	2.42	0.54	0.156	5.6	0.37	0.25
7	0.97	0.19	0.059	2.0	0.40	0.23
Recovery after sonication, %	107	75	100	104		
Recovery on the gradient, %	84	85	113	138		

cytochrome *c* reductase ratio decrease sharply in the upper 2–3 fractions of the gradient. However, the decrease is less pronounced in the case of the latter. NADPH-cytochrome *c* reductase activity is decreased in comparison to the NADH-cytochrome *c* reductase in the lower part of the gradient (Fig. 10 *a* and Table VI). NADPH-cytochrome *c* reductase activity in fraction 7 may be as high as in most other fractions of the gradient, but the PLP content is usually too low to be accurately quantitated. When fractions 5, 6, and 7 are diluted to approximately 0.25 M sucrose and centrifuged for 16 hr at 105,000 *g*, over 75% of the NADPH-cytochrome *c* reductase activity is recovered in the pellet with almost all the PLP. All reductases are less active in the pellet than in the unfractionated membranes.

The distribution of the reductases in the newborn is similar to that in the adult (Fig. 10 *a*). In phenobarbital-treated rats the differences between the NADH-reductases and NADPH-reductase are somewhat less striking, particularly after 6 days of treatment (Fig. 10 *b*).

Cytochrome b_5 and Cytochrome P-450

In the adult, cytochrome b_5 and cytochrome P-450 (dithionite reduced) have contrasting distributions, shown in Fig. 11 and Table VII. The distribution of cytochrome b_5 is similar to that of NADH-cytochrome *c* reductase. There is a concentration of this cytochrome in the lower part of the gradient and a progressive decrease in concentration in the smaller fragments at the top. Cytochrome P-450, on the other hand, has a distribution resembling that of NADPH-cytochrome *c* reductase activity. Concentrations are always low in the pellet and sometimes in fraction 1 as well (Fig. 11). In Fig. 12 the difference spectra of fractions 1 and 6 are shown to illustrate the differences in cytochrome distribution. The ratio of cytochrome b_5/cytochrome P-450 in various subfractions is shown on Table VII. In contrast to the

sharp decrease in the NADH/NADPH reductase ratios at the top of the gradient (Table VI), the ratio of the two cytochromes decreases gradually over the entire range of the gradient. Similar patterns are observed in phenobarbital-treated animals and in the 3-day-old animal. Absolute concentrations in the latter are much lower and the results are not shown on the figure.

In the NADPH-linked microsomal electron-transport chain, cytochrome *c* appears to be a direct electron acceptor of the flavoprotein (38, 39), and CO-binding of dithionite-reduced cytochrome P-450 is a reflection of enzyme amount and requires only the presence of the cytochrome. only in the lower part of the gradient and was scarcely detectable in the small fragments of the upper part of the gradient. The contrast between dithionite- and NADPH-reduced cytochrome P-450 in different portions of the gradient is illustrated in Fig. 13 and Table VIII.

The discrepancy between the distribution of cytochrome P-450 measured after dithionite reduction and the distribution of the NADPH-reduced cytochrome suggested the possibility that an intermediate component might be rate-limiting for the latter reaction. The NADPH-linked lipid peroxidation was therefore investigated as a reflection of this hypothetic intermediate between the flavo-

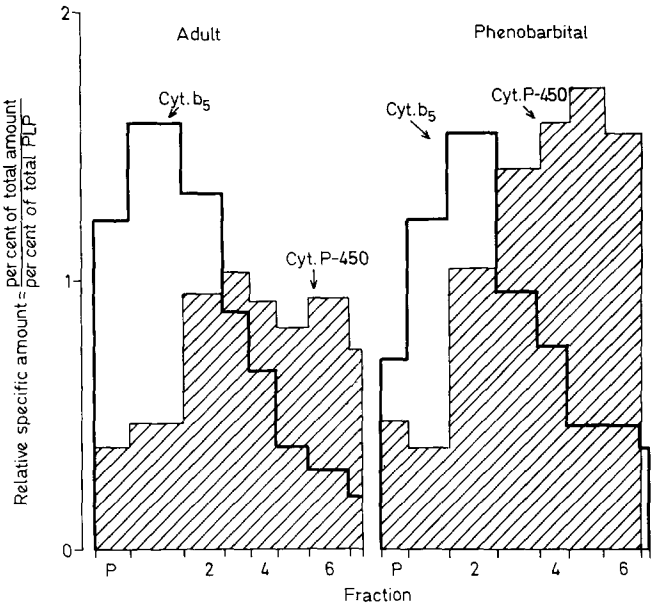


FIGURE 11 Distribution of cytochrome b_5 and cytochrome P-450 in subfractions. Adult (left) and phenobarbital-treated adult (8 mg/100 g per day) for 3 days (right). Distribution represented as in Fig. 10.

Reduction of cytochrome P-450 by NADPH, however, is dependent upon intermediate components within the chain. These include a flavoprotein and an uncharacterized component which is also involved in the lipid peroxidation reaction (18, 40). Disruption of linkages within the chain, or loss of essential cofactors, might therefore decrease the amount of the cytochrome that can be reduced by NADPH. In order to investigate this possibility, the concentration of cytochrome P-450 was determined after reduction with NADPH, as well as with dithionite as above. NADPH reduction in total rough microsomes results in a much lower peak at 450 mμ in comparison with the dithionite-reduced sample. The NADPH-reduced cytochrome P-450 was found in high concentration protein and the cytochrome. The lipid peroxidating activity follows a distribution similar to that of NADPH-reduced cytochrome P-450 but quite distinct from that of NADPH-cytochrome *c* reductase and dithionite-reduced cytochrome P-450 (Table VI).

Glucose-6-Phosphatase Distribution

The interpretation of glucose-6-phosphatase distribution is complicated by frequently inconsistent recovery data. The activity is generally high in the pellet and in the lower part of the gradient and is decreased by as much as 50% in the upper 2 or 3 fractions. However, the recovery of the enzyme in 0.29 M sucrose is only about 60% of the recovery in 1.32 M sucrose. It is therefore doubtful

that there is a substantial decrease in glucose-6-phosphatase in the smaller vesicles that is not accounted for by greater enzyme lability in low sucrose concentrations. Variations in enzyme activity of similar or greater magnitude are also noted in the range of pH between 5.6 and 7.6, and with changes in dilution.

TABLE VII

Enzyme Composition of Subfractions: Cytochrome b_5 and Cytochrome P-450

Difference spectra were determined in unsonicated and sonicated rough microsomes from 50 mg liver/ml. The pellet was suspended in 4 ml water (per pellet), and the undiluted fractions were prepared as described in Fig. 12. Enzyme amounts are expressed per ml of the undiluted rough microsomes and fractions. Enzyme ratios are based upon a value of 1.00 in the sonicated rough microsomes.

	Cytochrome b_5	Cytochrome P-450	Cytochrome b_5 / Cytochrome P-450
	mμmoles/ml		
Rough microsomes	4.03	2.80	1.46
Rough microsomes, sonicated	2.34	2.36	1.00
Pellet	0.238	0.077	3.13
Fraction 1	0.360	0.110	3.30
2	0.275	0.204	1.36
3	0.128	0.154	0.84
4	0.088	0.126	0.71
5	0.062	0.137	0.46
6	0.059	0.198	0.30
7	0.022	0.044	0.51
Recovery with sonication, %	58	84	
Recovery on the gradient, %	98	84	

DISCUSSION

The aim of the current series of experiments is to investigate the structural organization of rough-surfaced membranes by fractionating small fragments of membrane produced by sonication. According to the unit membrane theory proposed by Danielli and Davson (41) and electron microscopically demonstrated by Robertson (42), all membranes have in common a continuous bimolecular layer of lipid completely covered by protein. An alternative hypothesis suggests that membranes are made up of globular (43) or repeating units (44, 45). The development of each of these theories must incorporate increasing evidence of heterogeneity among various types of membranes and within individual membranes. The membranes of each subcellular organelle appear to have a distinctive chemical (46) as well as enzymic composition (47). They vary from one another in thickness (48–50), permeability (51, 52), and morphologic appearance after lipid extraction (53). The lipid and protein components within the same membrane also appear to differ in their turnover (54).

In total microsomes, rough- and smooth-surfaced membrane fractions differ from one another in synthetic function, composition, and turnover (1, 2, 21, 23). The isolated rough membrane fraction offers advantages as a starting material for the study of membrane structure because the attached ribosomes identify its origin from predominantly a single structural component of the cell. Rough membranes, although morphologically uniform, in turn exhibit heterogeneity with respect to certain enzymes after fractionation on a sucrose

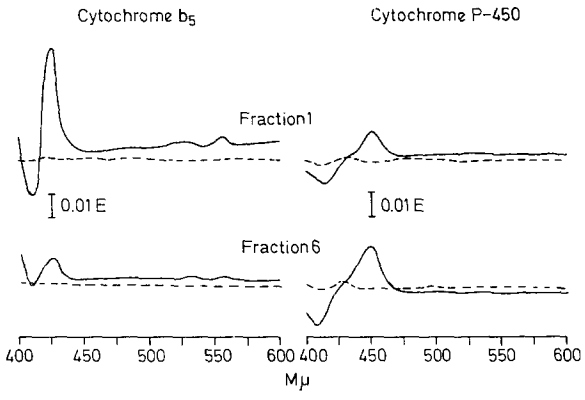


FIGURE 12 Difference spectra of cytochrome b_5 and P-450 in fractions 1 and 6. Both cuvettes contain 2.25 ml of the sample and 50 mM phosphate buffer pH 7.5 in a total of 2.5 ml. Cytochrome b_5 was determined after the contents of one cuvette were reduced with NADH in a final concentration of 0.25 mM. Cytochrome P-450 was determined after a few grams of $Na_2S_2O_4$ were added to both cuvettes and the contents of one cuvette were bubbled with CO (O_2 free). Base line traces are shown as dashed lines.

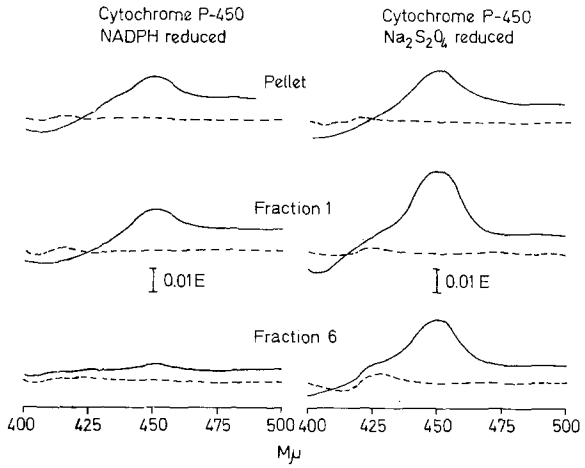


FIGURE 13 Difference spectra of cytochrome P-450 in the pellet, fraction 1 and fraction 6. The pellet was suspended in 4 ml water. Both cuvettes contain 2.25 ml of the sample and 50 mM phosphate buffer pH 7.5 in a total volume of 2.5 ml. Reduction was obtained by adding either NADPH to a final concentration of 0.25 mM or a few grams of $Na_2S_2O_4$ to both cuvettes and then bubbling one cuvette with CO for 30 sec. Base line traces are shown as dashed lines.

TABLE VIII

Comparison of Cytochrome P-450 Reduced with NADPH or $Na_2S_2O_4$ in Subfractions

Difference spectra are determined in unsonicated and sonicated rough microsomes from 50 mg liver/ml. The pellet is suspended in 4 ml water (per pellet), and the undiluted fractions are prepared as described in Fig. 12.

Sample	NADPH-reduced $OD_{450-490}$	$Na_2S_2O_4$-reduced $OD_{450-490}$	NADPH-reduced / $Na_2S_2O_4$-reduced
Rough microsomes, unsonicated	0.0050	0.0205	0.23
Rough microsomes, sonicated	0.0035	0.0165	0.21
Pellet	0.0085	0.0120	0.71
Fraction 1	0.0080	0.0215	0.37
2	0.0070	0.0320	0.22
3	0.0020	0.0220	0.09
4	0.0020	0.0190	0.11
5	0.0020	0.0215	0.09
6	0.0015	0.0165	0.09

gradient (8). In the present study, sonication produced much smaller fragments than did homogenized material, and with different properties. Heterogeneous patterns of enzyme distribution within vesicles of very small dimension suggest some tentative comments regarding the organization of proteins within the rough-surfaced membrane.

Possible arrangements of enzymes within membranes in general can be considered in the following three categories:

1) Individual enzymes located within the membranes in an entirely random fashion.

2) Components of a single electron-transport chain or enzymes performing related functions grouped in sequence, each assembly located singly in the membrane.

3) Collections of identical enzyme groups located in specialized sections of membrane.

The first model seems the least probable. The protein-lipid associations in the membrane are generally considered structural in the sense of favoring specific arrangements of enzyme molecules which already would be nonrandom (10, 55). This has been postulated to be the case in mitochondrial membranes where adjacent enzymes on the electron-transport chain may be grouped as described in model 2. The third possibility, involving areas of membrane specialized in composition and function, might be exemplified by the inner and outer membranes of the mitochondria (15). Indeed, the successful separation of these two types of membrane by sonic disruption followed by sucrose gradient centrifugation, suggested the use of these techniques in the present study.

In the case of the rough membrane, structure 1 would result in fragments of similar composition with the exception of fragments small enough to contain only a few protein molecules. With structures 2 and 3, a particle might be concentrated with respect to a single group of related enzymes. The distinction between 2 and 3 could then be made on the basis of the size of the particle that is

concentrated in respect to an enzyme assembly. If these particles were of a characteristic size or density, suitable conditions for fractionation would exist.

The present experiments focus on the two groups of related membrane enzymes that make up the NADH- and NADPH-linked sequences (Fig. 14). The results indicate that, in sonicated rough membranes, fractions containing small vesicles differ from those containing large vesicles in a manner which suggests a certain degree of separation of NADH-linked enzymes from the NADPH-linked group. Thus NADH-cytochrome *c* reductase, NADH-ferricyanide reductase, and cytochrome b_5 are all similar in distribution. They appear to be concentrated in the larger, more rapidly sedimenting particles and are decreased in the small, slowly sedimenting particles. In the small vesicles, major

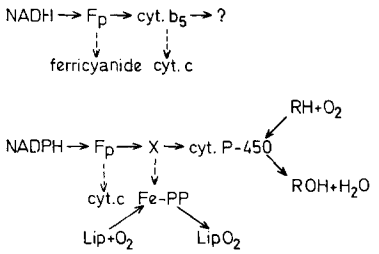


FIGURE 14 Tentative representation of NADH- and NADPH-linked electron-transport enzymes in liver microsomes.

loss or dissociation of one of the intermediates within this electron-transport chain seems unlikely in view of the very small difference in distribution between the two NADH reductases, one of which requires a complex assembly of enzymes. The good recovery of NADH-cytochrome *c* reductase after sonication is similarly an argument against more than slight denaturation or dissociation within the chain.

The maximal reduction of cytochrome b_5 with NADH may require the structural integrity of the electron-transport chain. The average loss of about 30% with sonication could be due to partial denaturation of the cytochrome, to dissociation of the chain, or to loss of other components or cofactors, making the reduction by NADH less complete. All these possibilities seem inconsistent with the good recoveries of NADH-cytochrome *c* reductase activities after sonication. This observation and the fact that loss of cytochrome b_5 (reduced with NADH) is relatively small, suggest that the NADH-linked electron-transport chains remain for the most part structurally intact and that they are concentrated in the larger vesicles at the lower part of the gradient.

The distribution of certain NADPH-linked enzymes differs from that of the NADH-linked group. Both NADPH-cytochrome *c* reductase and cytochrome P-450, reduced by dithionite, are somewhat decreased in the larger fragments and are apparently concentrated in the upper part of the gradient. However, a proportion of the enzyme molecules concentrated at the top of the gradient may no longer be part of an intact chain. As both determinations estimate a single protein, the possibility of disruption of linkages within the chain is not excluded by a high concentration of the individual enzymes. Indeed, the reduction of cytochrome P-450 with NADPH suggests this possibility. By this reaction cytochrome P-450 is present almost entirely in the rapidly sedimenting fractions and is almost undetectable in the smaller fragments. The NADPH-linked lipid peroxidating system also follows a distribution similar to that of enzymatically reduced cytochrome P-450. It is possible that an intermediate component of the reaction sequence between the flavoprotein and the cytochrome (labeled X on Fig. 14) becomes a rate-limiting factor to both reactions. An alternate possibility is that only some molecules of cytochrome P-450 are linked to NADPH whereas the remainder may participate in other reaction sequences.

If two electron-transport chains have indeed been separated to some extent, the sizes of the fragments in which they are localized may help in distinguishing among certain models of membrane organization. The smallest vesicles seen on electron micrographs of the upper third of the gradient have a diameter of about 300 A. It is of interest that Robertson considers this diameter the minimum vesicle diameter compatible with the unit membrane theory and the smallest size observed in sections of tissues (56). The volume of a 60 A thick shell with a 300 A outside diameter is 1.25×10^4 $m\mu^3$. In estimating the protein content of this vesicle, the corrections for an approximately 30% lipid content and a protein specific density of about 1.5 tend to cancel out. If this vesicle is calculated to contain only one protein molecule, the molecular weight of the protein would be approxi-

mately 7.5×10^6. A vesicle of this small size would be likely to include relatively few individual enzymes and even fewer enzyme assemblies. If a substantial contribution by "structural protein" is taken into account, this further reduces the possible number of enzyme molecules. The volume of a larger vesicle of 1100 A diameter, representative of the lower part of the gradient, is 2×10^5 mμ^3. By the same calculation as above, this vesicle could contain a protein of 1.2×10^8 molecular weight. This particle must be able to readily accommodate many enzyme assemblies. A concentration of NADH-linked enzymes in these vesicles suggests a nonrandom collection of functionally related groups of enzymes.

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