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MOLECULAR COMPOSITION OF THE SUBMICROSOMAL MEMBRANE LIPID OF RAT BRAIN

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ABSTRACT

Rough-surfaced and light and heavy smooth-surfaced microsomes were isolated from rat brain by means of discontinuous sucrose gradient centrifugation. Electron microscopically, the rough-surfaced microsomes were characterized by vesicles with ribosomes and the light and heavy smooth-surfaced microsomes by fairly homogeneous membrane features without ribosomes.

The rough-surfaced microsomal membranes were distinguished by the absence of glycolipids, such as ganglioside, cerebroside, and sulfatide. Cerebroside was exclusively recovered in the light smooth-surfaced microsomal membranes. Ganglioside and Na,K-ATPase were contained in larger amounts in the heavy smooth-surfaced microsomal membranes than in the light smooth-surfaced microsomal membranes in terms of protein content.

Among the three submicrosomal membranes, cholesterol and phospholipid were found in the largest amounts in the light smooth-surfaced microsomal membranes, where the molar ratio of cerebroside-cholesterol-phospholipid was about 1:10:10.

The membranes of rough- and smooth-surfaced microsomes were very similar in regards to the composition of phospholipid classes, although the fatty acid composition of the former contained a greater proportion of unsaturated fatty acids than that of the latter.

When the membrane proteins were analyzed by sodium dodecyl sulfate gel electrophoresis, some differences were observed between the light and heavy smooth-surfaced microsomal membranes.

Although differences in the properties of biological membranes can be linked to differences in lipid composition, our knowledge of the biological significance of lipid constituents of membranes is very limited. In particular, no convincing studies on the distribution of glycolipids in neuronal membranes have been made, due to the inherent anatomical complexity of the brain. Postmitochondrial particles, though heterogeneous (3), have so far been referred to as "microsomes" of the brain, and most studies of their neurobiological significance have utilized such heterogeneous fractions (5, 7, 12, 21, 35). Thus, to clarify unequivocally the distribution of glycolipids in neuronal

membranes and their significance, it is a prerequisite to prepare pure neuronal and membrane fractions. From this point of view, we have studied the chemical composition of the isolated neuronal perikaryon, which clarified some chemical characteristics of the neuron (28, 29). In contrast to the presence of abundant endoplasmic reticulum and well-preserved plasma membrane, neither cerebroside nor sulfatide was detected in the cell body; an unexpectedly small amount of ganglioside was present, though these lipids had previously been shown to be constituents of microsomes in the brain. These observations imply that cerebroside and sulfatide are not neuronal constituents and, moreover, that they may be nonmicrosomal lipids in many kinds of cells, and that ganglioside may be diffusely localized over the whole neuronal plasma membrane. On the other hand, significant amounts of cerebroside and sulfatide have been observed in isolated neuronal perikarya by Norton and Poduslo (18) and Hamberger and Svennerholm (11).

These experimental findings led us to investigate the distribution of lipids, including ganglioside, cerebroside, and sulfatide, as well as Na,K-ATPase, in submicrosomal membranes, using electron microscopically well-defined materials. Some unusual chemical features of submicrosomal membranes of the rat brain are reported.

MATERIALS AND METHODS

Isolation of Submicrosomal Membranes

The basic procedure followed the slightly modified method of Rothschild (20) and Peters (19), originally developed for the isolation of smooth- and rough-surfaced microsomes of the liver. Five to ten Wistar male rats, weighing 200 g on average, were used in each experiment. Under ether anesthesia, the brain was perfused through the left ventricle with 50 ml of saline until the red color of the eyes faded. The rat was then decapitated, and the whole brain was rapidly removed and placed in ice-cold saline. The cerebrum, after being freed from the cerebellum and the brain stem, was minced with tweezers and then homogenized in 4 vol of 0.88 M sucrose solution with a Teflon-glass homogenizer having a clearance of about 0.25 mm. This homogenization procedure was carried out carefully at a constant rate of one stroke per minute to ensure consistent yield and quality of the membrane fractions. The homogenate was centrifuged at 25,000 *g* for 20 min. The supernate was mixed with an equal volume of 1.76 M sucrose, and 2 ml of the mixture was carefully overlaid with 7 ml of 1.23 M sucrose and 1.5 ml of 0.15 M sucrose successively, then centrifuged at 105,000 *g* for 16 h in Beckman fixed angle rotors. After 16 h, a cloudy upper phase at the gradient boundary between 0.15 and 1.23 M sucrose, a slightly opalescent intermediate phase between the upper phase and the pellet, and a clear yellow pellet were observed. These two phases and the pellet were designated as light smooth-surfaced, heavy smooth-surfaced, and rough-surfaced microsomal membranes, respectively. Light and heavy smooth-surfaced microsomal membranes were removed separately with a J pipette, diluted with 3 vol of cold water, and sedimented as pellets by centrifugation at 105,000 *g* for 2 h. The inner walls of the test tubes which contained the three different microsomal membranes as pellets were rinsed with cold water and wiped with soft paper. Then, the membrane fractions were homogenized in cold water and centrifuged at 105,000 *g* for 90 min. This washing procedure was repeated two times. The pellets thus obtained were suspended in a given volume (5 ml) of water by thorough homogenization and subjected to chemical analyses.

Electron Microscopy

Submicrosomal membranes were obtained as pellets, as described above, except that water was replaced by 0.32 M sucrose for washing. The microsomal pellets were fixed for 8 h in 1% OsO_4 in Millonig's phosphate buffer at pH 7.3 in the cold, then dehydrated by increasing the concentration of ethanol. After immersion in propylene oxide, the pellets were embedded in Epon. Ultrathin sections were obtained from the top, middle, and bottom parts of the pellets and stained with uranyl acetate and lead citrate. After carbon impregnation *in vacuo*, specimens were examined under a Hitachi HU-11B electron microscope.

Chemical Analyses

The analytical data presented in this paper are averages of more than ten different preparations of submicrosomal membranes, unless otherwise indicated. Protein content was determined by the method of Lowry et al. (16). RNA was extracted by the procedure of Fleck and Munro (9) as modified by Steele et al. (26) and was determined by the orcinol reaction (6). Na,K-ATPase activity was assayed in a medium containing 5 mM Tris-ATP, 100 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.4, in the presence or absence of 1.5 mM ouabain. The reaction was terminated by the addition of trichloroacetic acid at a final concentration of 6%. Released inorganic phosphate was determined by the method of Fiske and Subbarow (8). The difference between the values in the absence and in the presence of ouabain was designated as the Na,K-ATPase activity and expressed in micromoles Pi released per hour per gram wet weight of tissues.

Lipid was extracted with 20 vol of chloroform-methanol (2:1, by volume) in a Teflon-glass homogenizer. After filtration, the extract was evaporated to

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dryness under an N_2 stream in a rotary evaporator. The dried material was dissolved in a given volume of chloroform-methanol (2:1) and substances soluble in this solvent were partitioned against water as described by Folch et al. (10). The upper and lower phases thus obtained were adjusted to 2 and 4 ml, respectively, and subjected to chemical analysis. Silica Gel-G (Merck, Darmstadt, W. Germany) plates (0.25 or 0.4 mm in thickness) were used for thin-layer chromatography (TLC) after activation for 90–120 min at 120°C. Two-dimensional TLC of total lipid classes in the lower organic phase was carried out in a mixture of chloroform-methanol-concd ammonia (13:7:1) in the first dimension followed by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1) in the second dimension. Cholesterol was measured by the method of Searcy and Bergquist (22), and the phosphorus of the total lipid by Bartlett's method (2). The individual phospholipids were separated on TLC plates as described by Skipski et al. (24), and determined as described by Keenan et al. (13), with materials scraped from the TLC plates. *N*-Acetylneuraminic acid (NANA) in the upper aqueous phase, taken as an indicator of ganglioside, was measured as described by Warren (32). Cerebroside was determined by a photodensitometric method as follows: Three different concentrations of total lipid in the lower organic phase and known concentrations of kerasine (cerebroside with nonhydroxy fatty acid) purified from bovine brain were carefully spotted in 5-mm bands on a Silica Gel-G plate and were developed in a mixture of chloroform-methanol-water (65:25:4). After development, the plate was sprayed with 3 ml of 50% H_4SO_4 and charred on a 2-kW hot plate at maximum temperature for 40 min. The densities of the charred spots were scanned with a Schoeffel spectrodensitometer model SD 3000 (Schoeffel Instrument Corp., Westwood, N. J.) at 565 nm with a slit width of 0.5 mm.

For gas-liquid chromatographic analyses of the fatty acid composition of phosphoglyceride, lipid was freshly extracted from submicrosomal membranes as described above, and fatty acids were methylated with sodium methoxide in dry methanol as described by Svennerholm (27). Chromatography was carried out at 160°C with a Shimazu model GC-4BM gas chromatograph using a glass column 1.5 m in length packed with 15% ethylene glycol succinate on Celite 545. The individual esters were identified by comparison with authentic samples or with the aid of a plot of log retention times.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis of the Membrane Proteins

The membrane fractions were dissolved in 10 mM sodium phosphate buffer, pH 7.0, which contained 1% each of SDS and β-mercaptoethanol. After dialysis against 10 mM sodium phosphate buffer, pH 7.0, which contained 0.1% each of SDS and β-mercaptoethanol, electrophoresis was carried out in 7.5% acrylamide gel containing 1% SDS by the method of Weber and Osborn (33). Gels were stained with Coomassie brilliant blue (33). The density of the destained gel was traced with a Joyce-Loebl microdensitometer equipped with a 620-nm filter (Joyce, Loebl & Co., Ltd., Gateshead, England).

RESULTS

Identification of Submicrosomal Membrane Fractions

Representative photographs of the three submicrosomal membrane fractions are shown in Fig. 1. The rough-surfaced microsomal membrane fraction (Fig. 1 *c*) was characterized by vesicles with ribosomes and free ribosomes. Larger particles, more electron dense, were also seen. In the light and heavy smooth-surfaced microsomal membrane fractions (Fig. 1 *a*, *b*), ribosomal particles and the electron-dense large particles were hardly present, but vesicular elements of various sizes were seen. The heavy smooth-surfaced microsomal membrane fraction appeared more homogeneous in regards to membrane structure than the light smooth-surfaced microsomal membrane fraction. In the latter fraction, mitochondria, synaptosomal debris, and myelin were seen as minor contaminants.

Components Analysis and Enzyme Activity of Submicrosomal Membranes

The amounts of protein, RNA, Na,K-ATPase, cholesterol, phospholipid, and lipid-bound NANA are summarized in Table I. The amount of protein recovered in the smooth-surfaced microsomal membranes was about three times as high as that in the rough-surfaced microsomal membranes. RNA was found in the highest amount in the rough-surfaced microsomal membranes, whereas a small amount was found in both types of smooth-surfaced microsomal membranes. This result was compatible with the electron microscope feature of the distribution of ribosomal particles in submicrosomal membrane fractions.

Na,K-ATPase activity was observed preponderantly in the smooth-surfaced microsomal membranes, whereas only a low activity was observed in the rough-surfaced microsomal membranes. Lipid-bound NANA was distributed in the same way as Na,K-ATPase activity, and was practically absent in the rough-surfaced microsomal membranes.

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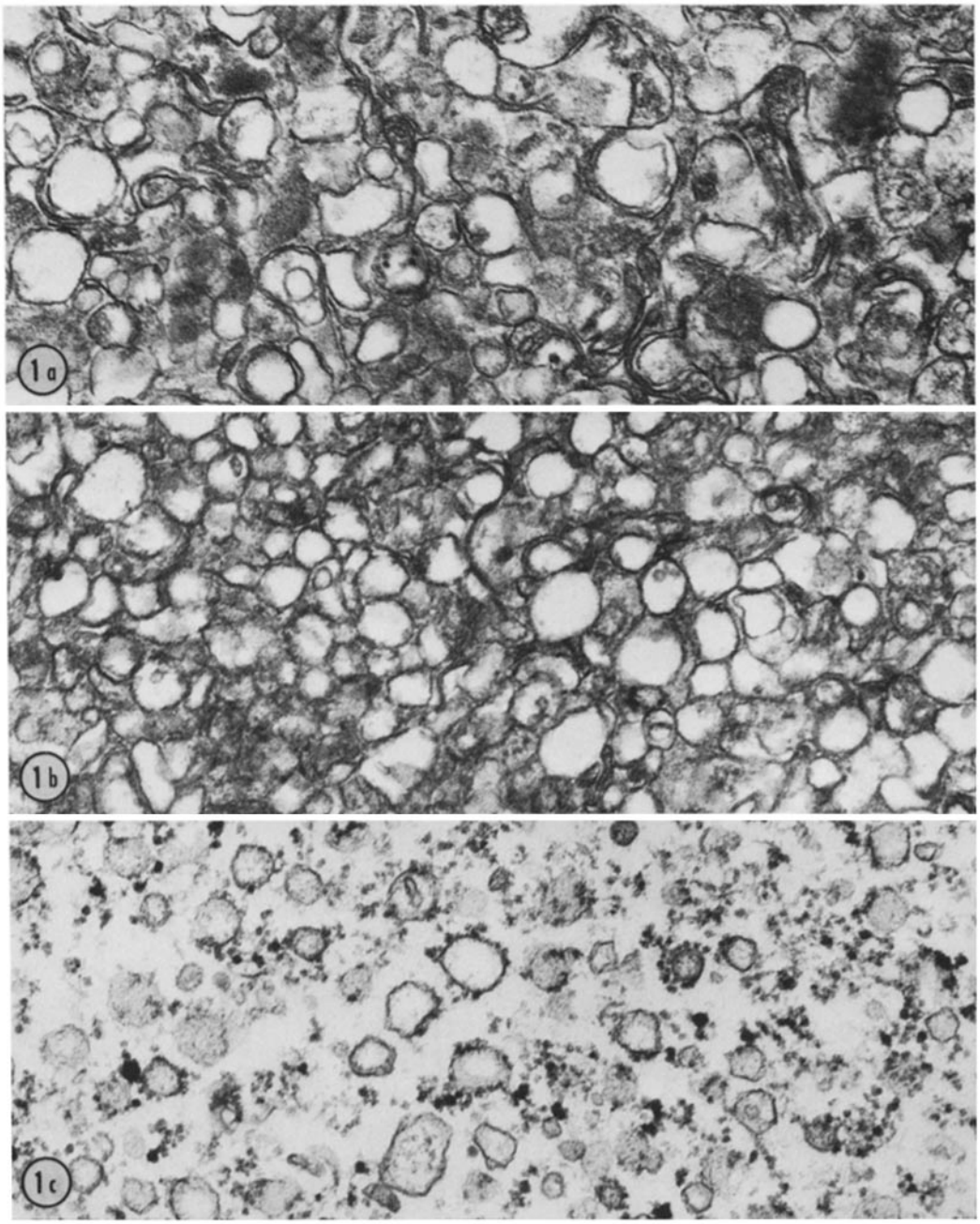

FIGURE 1 *a* Electron micrograph of light smooth-surfaced microsomes. These microsomes consist of membranous vesicles of a more heterogeneous appearance than those of heavy smooth-surfaced microsomes in regards to shape, size, density, and their content in the vesicles. × 50,000.

FIGURE 1 *b* Electron micrograph of heavy smooth-surfaced microsomes. The microsomes are more homogeneous than the light smooth-surfaced microsomes. × 50,000.

FIGURE 1 *c* Electron micrograph of rough-surfaced microsomes. Most of the vesicles are attached to ribosomes. Bodies denser and larger than ribosomes are seen besides many free ribosomes. × 50,000.

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TABLE I

Amounts of Protein, RNA, Na,K-ATPase, and Lipid Classes in Rat Brain Submicrosomal Membrane Fractions

Submicrosomal membranes	Protein		RNA	Na,K-ATPase	
	mg/g wet wt of tissue	Recovery*	μg/g wet wt of tissue	μmol Pi released/h/g wet wt of tissue	Recovery
		%			%
Rough-surfaced	1.4	1.3	233	2.5	0.3
Heavy smooth-surfaced	3.5	3.2	62	54.4	6.6
Light smooth-surfaced	3.4	3.1	14	36.3	4.4
Total	8.3	7.6	309	93.2	11.3

Submicrosomal membranes	Cholesterol		Phospholipid		Lipid-NANA	
	μg/g wet wt of tissue	Recovery	μg/g wet wt of tissue	Recovery	μg/g wet wt of tissue	Recovery
		%		%		%
Rough-surfaced	27	0.2	95	0.3	0	0.0
Heavy smooth-surfaced	565	3.4	1,749	4.6	26	6.5
Light smooth-surfaced	1,679	10.1	2,922	7.7	31	7.7
Total	2,271	13.7	4,766	12.6	57	14.2

* Recovery of components with respect to brain homogenate.

Both cholesterol and phospholipid were recovered in large amounts in the smooth-surfaced microsomal membranes, and in the light smooth-surfaced microsomal membranes, especially, a considerably higher amount of cholesterol was recovered.

Content of Lipid Classes in the Submicrosomal Membranes

Lipid classes in each submicrosomal membrane are demonstrated by thin-layer chromatography in Fig. 2. Cerebroside and sulfatide were barely detectable in the rough-surfaced microsomal membranes. In a striking contrast, these glycolipids were major constituents of the light smooth-surfaced microsomal membranes. In the heavy smooth-surfaced microsomal membranes, those glycolipids were occasionally detected in small amounts. Cholesterol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine were detected in all three submicrosomal membranes. In addition, sphingomyelin was observed in the smooth-surfaced microsomal membranes.

The content of lipid-bound NANA, an indicator of ganglioside, is shown in Table II. The average value per nanomole of phospholipid phosphorus was 4×10^{-2} nmol in the heavy smooth-surfaced microsomal membranes and 2×10^{-2} nmol in the light smooth-surfaced microsomal membranes. Lipid-bound NANA was negligible in the rough-surfaced microsomal membranes. The value of 0.1×10^{-2} nmol in preparations 3 and 4 of the rough-surfaced microsomal membranes was nearly at the lowest limit of spectrophotometric measurement with the amount of the material used. Thus, it is clear that the ganglioside content of the rough-surfaced microsomal membranes is less than 1/20 and 1/40 of those in the light and heavy smooth-surfaced microsomal membranes, respectively. It should be noted that the content of lipid-bound NANA per phospholipid phosphorus was approximately two times higher in the heavy smooth-surfaced microsomal membranes than in the light smooth-surfaced microsomal membranes.

Table III shows that a large amount of cerebroside was found in the light smooth-surfaced microsomal membranes. Despite the large amount of lipid subjected to analysis, as indicated in the footnote of Table III, no cerebroside was detected in the rough-surfaced microsomal membranes. The trace amounts of cerebroside detected in a few preparations of the heavy smooth-surfaced micro-

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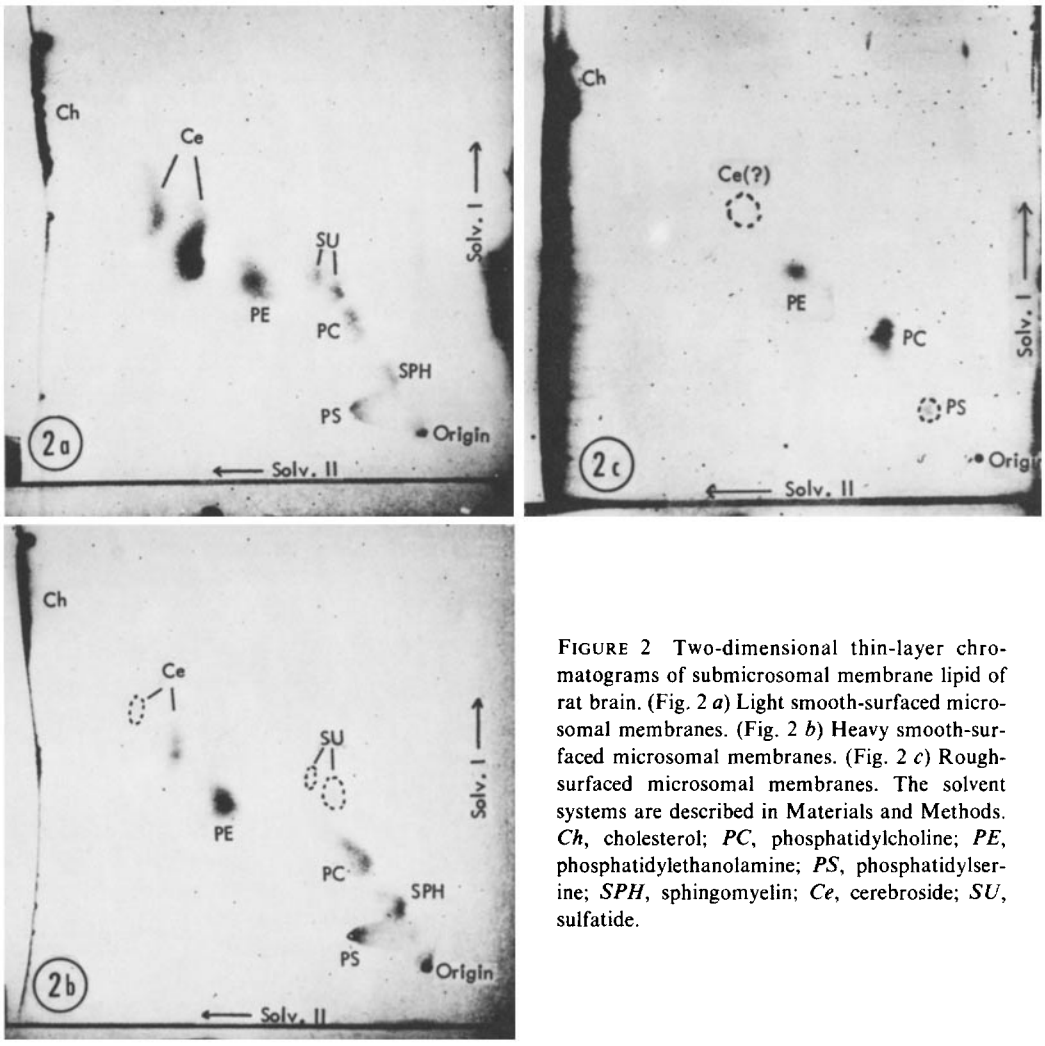

FIGURE 2 Two-dimensional thin-layer chromatograms of submicrosomal membrane lipid of rat brain. (Fig. 2 *a*) Light smooth-surfaced microsomal membranes. (Fig. 2 *b*) Heavy smooth-surfaced microsomal membranes. (Fig. 2 *c*) Rough-surfaced microsomal membranes. The solvent systems are described in Materials and Methods. *Ch*, cholesterol; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *SPH*, sphingomyelin; *Ce*, cerebroside; *SU*, sulfatide.

TABLE II

Lipid-Bound NANA Content of Rough- and Smooth-Surfaced Microsomal Membranes

Submicrosomal membranes	Preparations			
	1*	2	3	4
Rough-surfaced	0.0‡	0.0	$0.1 \times 10^{-2}>$	$0.1 \times 10^{-2}>$
Heavy smooth-surfaced	3.7×10^{-2}	4.0×10^{-2}		
Light smooth-surfaced	2.7×10^{-2}	1.8×10^{-2}	1.8×10^{-2}	

* The numbers indicate different preparations of submicrosomal membranes.

‡ Values are expressed as nanomoles of lipid-bound NANA per nanomole of phospholipid phosphorus.

somal membranes were probably due to slight contamination by the light smooth-surfaced microsomal membranes.

As shown in Table IV, all three membrane fractions had similar phospholipid compositions, except that the sphingomyelin content was very low in the rough-surfaced microsomal membranes. Phosphatidylcholine and phosphatidylethanola-

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TABLE III

Cerebroside Content of Submicrosomal Membranes

	Submicrosomal membrane preparations*						
	Rough-surfaced		Heavy smooth-surfaced			Light smooth-surfaced	
	1	2	1	2	3	1	2
	52.4	78.6	27.0	28.8	57.6	44.0	64.2
Cerebroside galactose‡	ND	ND	0.03	Trace	Trace	0.12	0.10

* Values for each preparation show the amount of lipid applied to TLC plates, expressed as nanomoles of phospholipid phosphorus.

‡ Values are expressed as nanomoles of cerebroside galactose per nanomole of phospholipid phosphorus.

ND, not detected.

TABLE IV

Phospholipid Composition of Rough- and Smooth-Surfaced Microsomal Membranes

	Percent lipid phosphorus			
Submicrosomal membranes	Phosphatidyletha-nolamine	Phosphatidyl-choline	Phosphatidylserine (+ phosphatidyl-inositol)	Sphingomyelin
Rough-surfaced	26.0	59.6	12.8	1.6
Heavy smooth-surfaced	26.9	51.7	13.7	7.7
Light smooth-surfaced	25.1	54.2	10.9	9.8

The values represent mean values of three separate submicrosomal membrane preparations (about 30 brains); three samples were analyzed in each case.

mine accounted for one-half and one-fourth, respectively, of the total phospholipid in all submicrosomal membranes.

The fatty acid composition of glycerophospholipid is shown in Table V. Palmitic, stearic, and oleic acids were major components in all three submicrosomal membranes. The content of arachidonic acid was higher in the rough-surfaced microsomal membranes than in the smooth-surfaced microsomal membranes. On the whole, the fatty acids of the former membranes appeared to be more unsaturated than those of the latter membranes.

SDS Gel Electrophoresis of Smooth-Surfaced Microsomal Membrane Proteins

As shown in Fig. 3, two minor bands (indicated by arrows) observed with the light smooth-surfaced microsomal membranes were faint or absent in the heavy smooth-surfaced microsomal membranes. Microdensitometric tracing of the gels revealed further dissimilarities. The proportions of

TABLE V

Fatty Acid Composition of Glycerophospholipids of Submicrosomal Membranes

	Fatty acids (wt %)		
	Submicrosomal membranes		
	Rough-surfaced	Heavy smooth-surfaced	Light smooth-surfaced
14:0	0.8	0.2	0.6
16:0	30.1	36.2	42.0
16:1	1.7	0.7	2.6
18:0	20.6	24.6	22.8
18:1	30.5	27.8	20.5
18:2	0.9	0.1	0.2
20:1	0.3	0.8	0.2
20:4	9.2	3.5	3.5
22:4	0.4	0.9	0.8
22:6	4.4	5.0	6.2
Unidentified	1.1	0.2	0.6
Saturated	51.5	61.0	65.4
Unsaturated	47.4	38.8	34.0

The values represent mean values of three separate submicrosomal membrane preparations.

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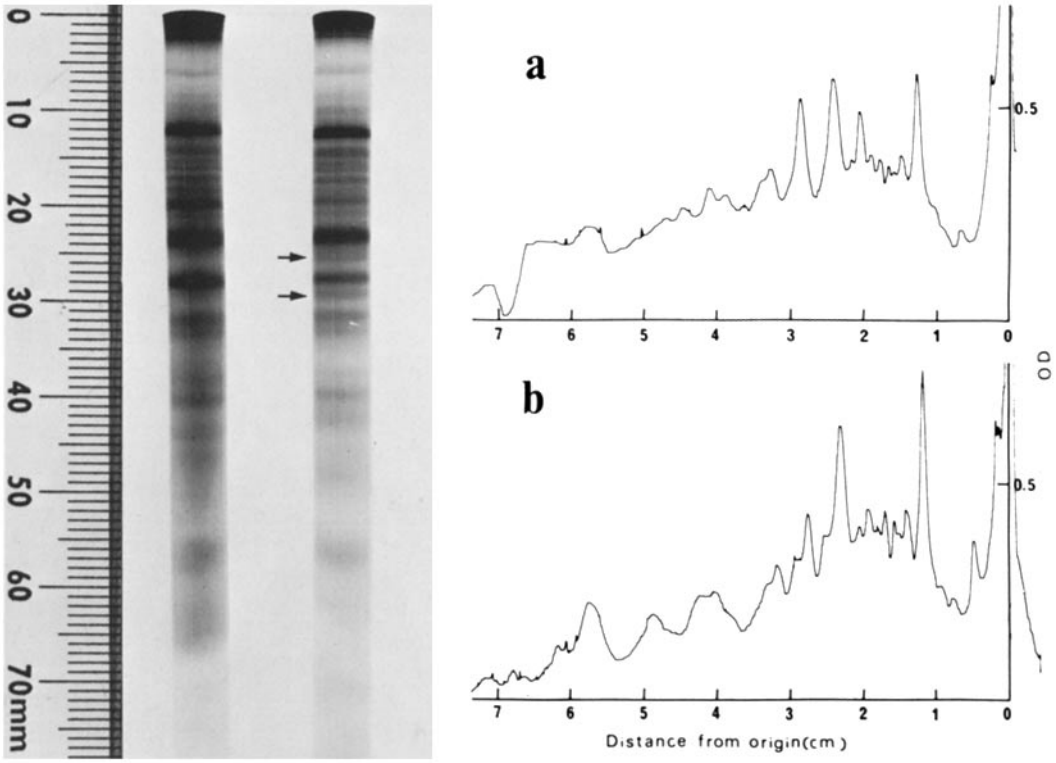

FIGURE 4

FIGURE 3 SDS-polyacrylamide gel electrophoresis of light smooth-surfaced (right) and heavy smooth-surfaced (left) microsomal proteins. The arrows indicate the protein bands which are absent in the heavy smooth-surfaced microsomes.

FIGURE 4 Densitometric tracing of SDS-polyacrylamide gel electrophoretogram of heavy smooth-surfaced (Fig. 4 *a*) and light smooth-surfaced (Fig. 4 *b*) microsomal proteins.

each protein band in the two membranes differed significantly (Fig. 4 *a*, *b*).

DISCUSSION

To isolate the submicrosomal membranes from rat brain, the procedure of Rothschild (20) and Peters (19), who worked with rat liver, has been applied in the present study. Three membrane fractions, light and heavy smooth-surfaced and rough-surfaced microsomal membranes, were obtained, and each of them was found to be fairly homogeneous by electron microscope observation.

Table VI and Fig. 5 show the distribution of individual lipids, the content of RNA, and the Na,K-ATPase activity in terms of micromoles per milligram of protein. It is clear that cerebroside is associated with the light smooth-surfaced microsomal membranes, but not with ribosome-bound membranes and the heavy smooth-surfaced microsomal membranes. Ganglioside is distributed only in ribosome-free membranes; the heavy smooth-surfaced microsomal membranes contained 1.5 times more of this lipid than the light smooth-surfaced microsomal membranes (Table VI). The present results provide the first evidence that glycolipids do not exist in ribosome-bound membranes. The view that membranes of rough- and smooth-surfaced endoplasmic reticulum are continuous has been proposed by some investigators (17, 34). If this concept is accepted, the present results on lipid compositions in submicrosomal membranes support our earlier suggestions (14, 28, 29) that glycolipids including ganglioside are not present inside the nerve cell perikaryon and that ganglioside is distributed on the neuronal plasma membrane. The amounts of lipid-bound NANA found in both smooth-surfaced microsomal membranes are within the range of values for unfrac-

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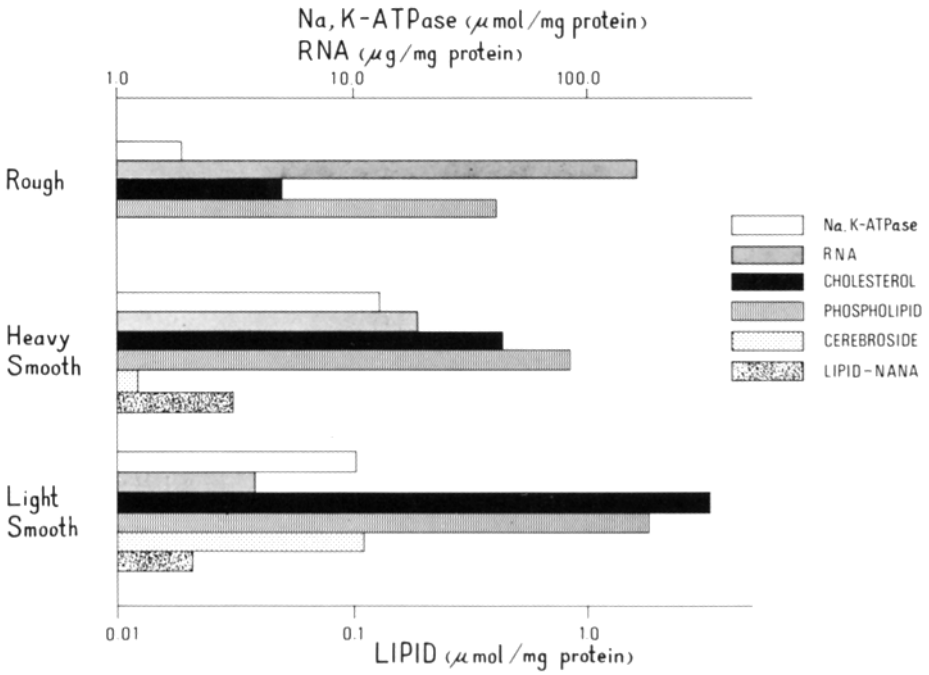

TABLE VI

Comparative Study of the Lipid Class Compositions in Rough- and Smooth-Surfaced Microsomal Membranes

Submicrosomal membranes	RNA (μg/mg protein)	Na,K-ATPase (μmol Pi released/h/mg protein)	Lipid class (μmol/mg protein)			
			Cholesterol	Phospholipid	Cerebroside	Ganglioside*
Rough-surfaced	166	1.8	0.05	0.31	ND	0.00
Heavy smooth-surfaced	18	15.5	0.42	0.70	0.0–0.02	0.03
Light smooth-surfaced	4	10.7	1.28	1.10	0.12	0.02

* Ganglioside content is expressed as micromoles of lipid-bound NANA.

tionated microsomes in the literature (approx. 0.023–0.052 μmol/mg protein) (1, 4, 15, 23, 25, 36, 37). Na,K-ATPase activity was also found exclusively in ribosome-free membranes. The activity in the heavy smooth-surfaced microsomal membranes is 1.5 times greater than that in the light smooth-surfaced microsomal membranes, in parallel with the distribution of ganglioside (Table VI).

In our preparations the molar ratio of cholesterol to phospholipid was about 0.2, 0.6, and 1 in the rough-surfaced and in the heavy and light smooth-surfaced microsomal membranes, respectively (Table VI). Considering that cholesterol may contribute to the stabilization of molecular architecture by strong van der Waals forces (30, 31) and that the fatty acids were less unsaturated in the smooth-surfaced microsomal membranes than in the rough-surfaced microsomal membranes (Table V), the membranes of the smooth-surfaced microsomes might be less fluid than those of the rough-surfaced microsomes. The phospholipid composition was not significantly different among the three submicrosomal membranes (Table IV). This kind of proportion of phospholipid may be a basic requirement for the biological functions of the membranes. In our previous work (28) the content of phosphatidylcholine was observed to decrease in the order of nerve cell perikarya, gray matter, and white matter. The present study has

shown that more phosphatidylcholine is contained in microsomal membranes than in nerve cell perikarya, suggesting that this lipid exists in greater quantities in the microsomal membranes than in the membranes of other cellular elements.

The results of SDS gel electrophoresis of the light and heavy smooth-surfaced microsomal membrane proteins revealed further dissimilarities in the chemical compositions of these microsomal membranes of the brain. These dissimilarities in protein components may be related to the differences in the lipid components discussed above.

Thus, in the present work we have determined the characteristic biochemical compositions of the individual submicrosomal membranes of the brain. In particular, the specific distribution of glycolipids has been determined. To clarify the biological significance of the lipids in submicrosomal membranes, further experiments are in progress.

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