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GANGLIOSIDES AND ACETYLCHOLINESTERASE IN ISOLATED MEMBRANES OF THE RAT-BRAIN CORTEX

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SUMMARY

The subcellular distribution of gangliosides was studied in the rat-brain cortex. In the primary fractions most of the gangliosides are in the microsomal and crude mitochondrial fractions, with the highest concentration in the light microsomes which contain mainly membranous components. After osmotic disruption of the synaptic complex, gradient techniques were used to purify the synaptic vesicles and to isolate the synaptic membranes. Two layers of synaptic membranes were found to contain the highest concentrations of gangliosides and acetylcholinesterase. Another layer of synaptic membranes poor in acetylcholinesterase had a low content of gangliosides. The lack of gangliosides and acetylcholinesterase in synaptic vesicles is emphasized, and the possible physiological significance of these complex acidic glycolipids in some neuronal membranes is discussed.

INTRODUCTION

The study of the subcellular localization of brain gangliosides is of considerable interest in view of their high concentration in the central nervous system, particularly in gray matter, and the various functional roles that have been attributed to these complex acid glycolipids. Gangliosides have been tentatively identified as receptors of tetanus toxin[1], associated with the maintenance of electrical activity and cation transport[2,3], and correlated with the distribution of (Na^+-K^+)-ATPase and acetylcholinesterase in subcellular fractions[4,5]. While most of the gangliosides have been found in the microsomal fractions, presumably in membrane fragments of dendrites[6], the use of mild homogenization procedures[7,8], which led to the isolation of nerve endings, has demonstrated the presence of gangliosides in these structures[6,9].

SEMINARIO, HREN AND GOMEZ[10] found a high concentration of gangliosides in two submitochondrial fractions which were previously shown to contain nerve endings

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rich in acetylcholine, acetylcholinesterase[11] and choline acetylase[12]. A low ganglioside content was found in the non-cholinergic nerve endings. The method of osmotic disruption of nerve endings, first developed in our laboratory[13], was used by BURTON *et al.*[14] to study the localization of gangliosides within the synaptic complex. The finding of an apparent association between acetylcholine and gangliosides in the fraction containing synaptic vesicles was interpreted as suggesting a functional role of these glycolipids in the binding and transport of this transmitter[14].

The present work was instituted to take advantage of new advances in the cell fractionation methods which have led: (a) to a purification of the synaptic vesicle fraction; (b) to the separation of a heavy microsomal fraction containing small nerve endings*; and (c) to the isolation of two types of nerve ending membranes (*i.e.* synaptic membranes). DE ROBERTIS *et al.*[15] applied osmotic shock to the crude mitochondrial fraction, followed by gradient centrifugation of the large subfraction M_1, to separate two layers containing synaptic membranes rich in acetylcholinesterase and (Na^+-K^+)-ATPase, and able to bind dimethyl-(+)-[^{14}C]tubocurarine and another layer poor in such enzymes and lacking other cholinergic properties.

It will be demonstrated here that brain gangliosides reach the highest concentration in the acetylcholinesterase-rich type of isolated synaptic membrane while synaptic vesicles are practically devoid of these acid glycolipids.

TABLE I

FRACTIONS AND SUBFRACTIONS OF BRAIN CORTEX

Fractions and subfractions	*Conditions*	*Ultrastructure*
A Primary fractions separated by centrifugation in 0.32 M sucrose at pH 6.2		
Nuclear (Nuc)	900 × *g*, 10 min, 2 washings	Nuclei, capillaries, myelin
Mitochondrial (Mit)	11 500 × *g*, 20 min 1 washing	Myelin, mitochondria, nerve endings
Microsomal (Mic)	100 000 × *g*, 60 min	Small nerve endings and microsomes
Supernatant (Sup)		Soluble fraction
B Subfractions of Mit after osmotic shock (1 g tissue per 10 ml redistilled water)		
M_1	20 000 × *g*, 30 min	Myelin, mitochondria and synaptic membranes
M_2	100 000 × *g*, 60 min	Synaptic vesicles and some membranes
M_3		Soluble
C Subfractions of M_1 on a gradient of sucrose after centrifugation of 50 000 × g, 2 h in the SW 25 rotor		
M_1 0.8	0.8 M sucrose	Myelin
M_1 0.9	0.9 M sucrose	Synaptic membranes and myelin
M_1 1.0	1.0 M sucrose	Synaptic membranes
M_1 1.2	1.2 M sucrose	Synaptic membranes
M_1 (p)		Mitochondria and some membranes
D Subfractions of M_2 on a gradient of sucrose after centrifugation of 50 000 × g, 1 h in the SW 39 rotor		
M_2A	0.32–0.5 M sucrose	Synaptic vesicles
M_2B	0.5 M sucrose	Membranes and few synaptic vesicles

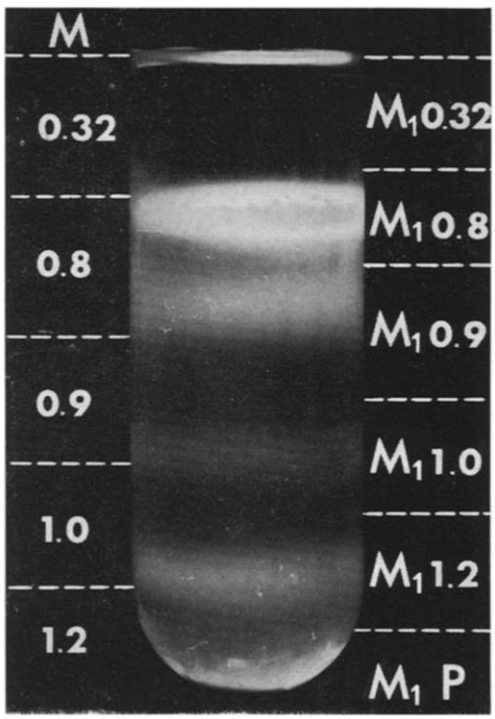
* K. KATAOKA AND E. DE ROBERTIS, unpublished results.

METHODS

Cell fractionation

For each experiment 8 adult Wistar rats were decapitated, and the brain cortex was separated in the cold room. The 3.8 — 4.2 g of tissue obtained were suspended in 4 volumes of 0.32 M sucrose at pH 6.2 and disrupted in a glass homogenizer provided with a loose teflon pestle (clearance 0.25 mm). The homogenate, diluted to 10% with 0.32 M sucrose, was submitted to a series of centrifugations to separate the primary fractions[11] (Table 1A). Sometimes, by an intermediary centrifugation, the microsomal fraction was divided into a heavy and a light fraction (*i.e.* mic-20 and mic-100)*. The crude mitochondrial fraction was submitted to an osmotic shock[13] and subfractionated as shown in Table 1B. To separate the synaptic membranes[15] subfraction M_1 was resuspended in 0.32 M sucrose (1 g tissue per 3.3 ml) and carefully layered on a sucrose gradient as indicated in Table 1C. The four layers and the pellet obtained are shown in Fig. 1.

Subfraction M_2 was purified further as described in TableID. Fig. 2 shows the layers obtained, namely M_2A and M_2B. The layers from the two gradients were separated by aspiration under visual control and sedimented as pellets at 100 000 $\times$ *g* for 1 h.



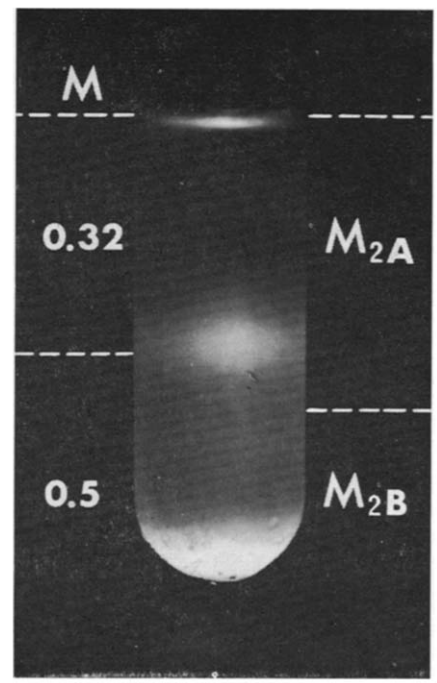


Fig. 1. Photograph of the gradient of M_1, made in a centrifuge tube of the SW 25 rotor according to the technique of DE ROBERTIS *et al.*[15], showing the sucrose concentrations and the various layers and pellet that are separated.

Fig. 2. Photograph of the gradient of M_2, made in a centrifuge tube of the SW 39 rotor, showing the sucrose concentrations and the layer M_2A and pellet M_2B obtained.

* K. KATAOKA AND E. DE ROBERTIS, unpublished results.

Biochemical determinations

All particulate fractions and subfractions were homogenized in distilled water. Lipids were extracted into 19 volumes of chloroform–methanol (2:1, v/v)[16], and the extracts were washed twice[17]. The upper phases were separated and dialyzed for 48 h with 15 changes of distilled water. Aliquots were evaporated under vacuum at 60° in a rotary evaporator and brought to dryness.

Gangliosides were assayed by determining *N*-acetylneuraminic acid (NANA) with the technique of WARREN[18] adapted to a sensitivity of 1–7 μg of NANA. The extracts were hydrolyzed for 2 h at 80°. Appropriate standards of NANA kindly supplied by Dr. L. SVENNERHOLM were used. Although it is known that some degradation of NANA takes place during hydrolysis[17], no correction factor was applied to our determinations. According to HESS AND ROLDE[17], to obtain the true absolute values, the data in Table II should be multiplied by 1.3.

Acetylcholinesterase was determined by the method of ELLMAN *et al.*[19], and proteins by that of LOWRY *et al.*[20].

Electron microscopy of the various particulate fractions was carried out with a Siemens Elmiskop 1 electron microscope, after fixation of the pellets in OsO_4, embedding in Epon 812 and thin sectioning.

Expression of results

Gangliosides are expressed in μg NANA per g fresh tissue, in percentage of total NANA recovered and in relative specific concentration (rel. spec. concn.).

TABLE II

GANGLIOSIDES AND ACETYLCHOLINESTERASE IN PRIMARY FRACTIONS, SUBMITOCHONDRIAL FRACTIONS AND IN THE GRADIENT OF M_1

Values for protein, NANA and acetylcholinesterase are the mean of three experiments. Absolute values per g tissue: protein, 126 mg; NANA, 679 μg and acetylcholinesterase, 219 μM acetylthiocholine hydrolyzed per h. For description of fractions see Table I.

Fractions and subfractions	*Protein*	*NANA*			*Acetylcholinesterase*
		μg/g	*%*	*rel. spec. concn.*	*(rel. spec. activity)*
Nuclear	7.3	20.2	3.2	0.44	0.95
Mitochondrial	44.6	264.0	41.7	0.93	0.88
Microsomal	31.4	336.0	53.0	1.69	1.47
Supernatant	16.7	13.1	2.1	0.13	0.56
Recovery (%)	104	93			
M_1	67.6	162.3	67.6	1.03	0.96
M_2	14.5	64.5	27.5	1.91	2.03
M_3	17.9	6.8	2.9	0.16	0.45
Recovery (%)	92	88.5			
M_1 0.32	4.4	1.9	1.6	0.37	—
M_1 0.8	19.7	26.7	23.1	1.17	1.68*
M_1 0.9	5.5	25.1	21.7	3.98	3.22*
M_1 1.0	6.5	21.9	18.9	2.92	2.13*
M_1 1.2	14.3	25.6	22.1	1.54	0.98*
M_1 (p)	49.6	14.5	12.6	0.25	0.15*
Recovery (%)	81	72			

* Data from DE ROBERTIS *et al.*[15].

$$\text{rel. spec. concn.} = \frac{\text{NANA \% recovered}}{\text{Protein \% recovered}}$$

To compare the content in gangliosides of all fractions with the total homogenate taken as one, a specific concentration ratio (spec. concn. ratio) is used:

$$\text{spec. concn. ratio} = \frac{\mu\text{g NANA per mg protein in fraction}}{\mu\text{g NANA per mg protein in total homogenate}}$$

RESULTS

Primary fractions (Table IA)

Table II shows the distribution of gangliosides in the various fractions and subfractions expressed in terms of NANA per g fresh tissue, in percentage and rel. spec. concn. Fig. 3 shows histograms in which the spec. concn. ratio values are plotted in relation to the percentage of protein. In the primary fractions 94.7% of NANA is recovered in the crude mitochondrial and microsomal fractions with very little in the nuclear fraction and in the supernatant. As percentage, the content of NANA is higher in the microsomes; however, the crude mitochondrial fraction also contains a considerable portion (41.7%). The rel. spec. concn. and the spec. concn. ratio show that only in the microsomes is there a definite concentration of gangliosides. The acetylcholinesterase activity follows approximately the same distribution as the gangliosides with the highest rel. spec. activity in microsomes.

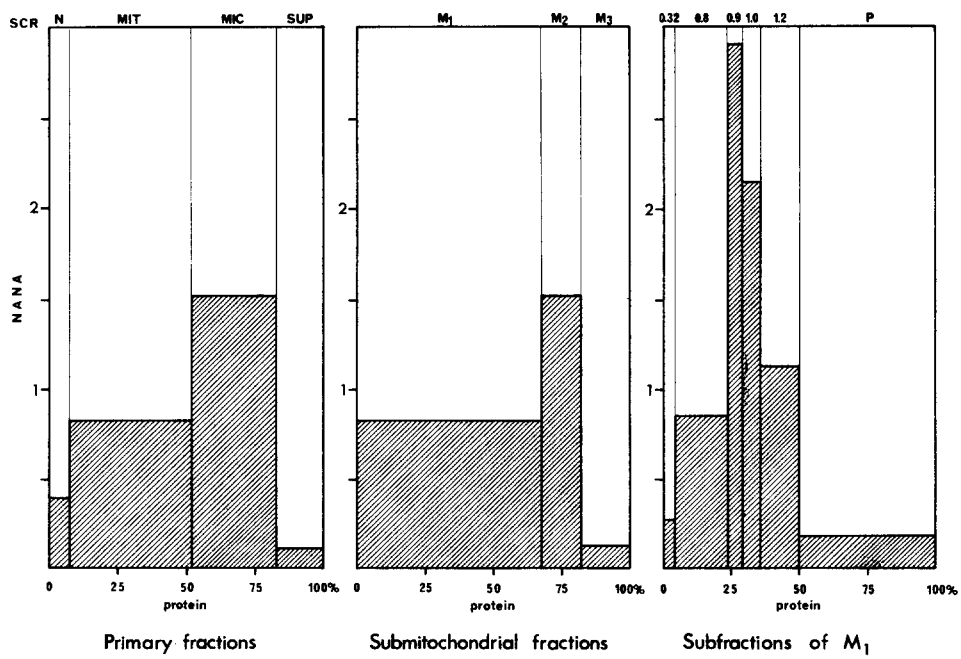


Fig. 3. Diagram showing the ganglioside content in specific concentration ratio (spec. concn. ratio) in relation to the percentage of protein for the primary fractions, the submitochondrial fractions after osmotic shock and the subfractions of M_1 on a density gradient. The rel. spec. concn. is defined in METHODS. In the homogenate the value of spec. concn. ratio is 1. The content of the various fractions and subfractions is given in Table I.

TABLE III

GANGLIOSIDES AND ACETYLCHOLINESTERASE IN HEAVY AND LIGHT MICROSOMES

Values are the mean of three experiments. Mic 20 was obtained by centrifugation of the supernatant of Mit at 20 000 × *g*, 30 min and Mic-100 by a centrifugation of the supernatant of Mic-20 at 100 000 × *g*, 60 min. Fraction Mic was considered as 100%.

	Protein (%)	*NANA*		*Acetylcholinesterase (rel. spec. activity)*
		%	*rel. spec. concn.*	
Microsomes-20	64.5	56	0.87	0.93
Microsomes-100	35.5	44	1.24	1.44
Recovery (%)	110	75		105

The morphological content of the microsomal fractions Mic-20 and Mic-100 (Table III) will be described and illustrated elsewhere*. The main component of Mic-20 is represented by small nerve endings and that of Mic-100 by membranous profiles of

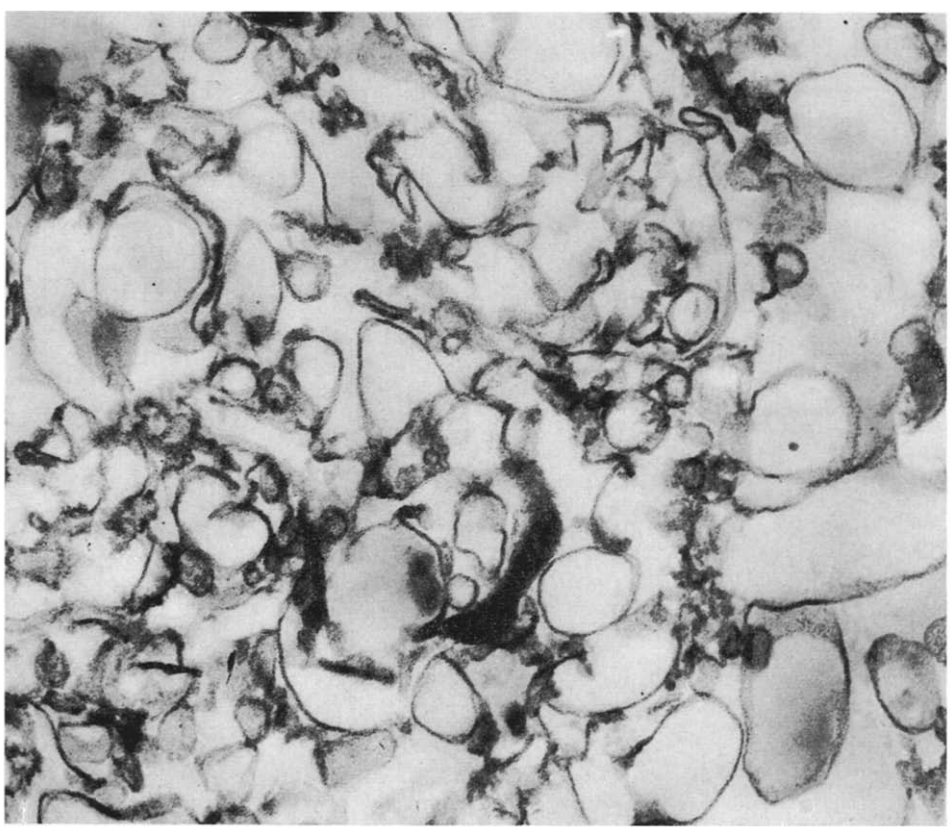


Fig. 4. Electronmicrograph of subfraction M_1 0.9 showing that it is mainly composed of membrane profiles having a size approximately similar to that of small nerve endings. This subfraction has the highest NANA rel. spec. concn. See Table II. × 60 000.

* K. KATAOKA AND E. DE ROBERTIS, unpublished results.

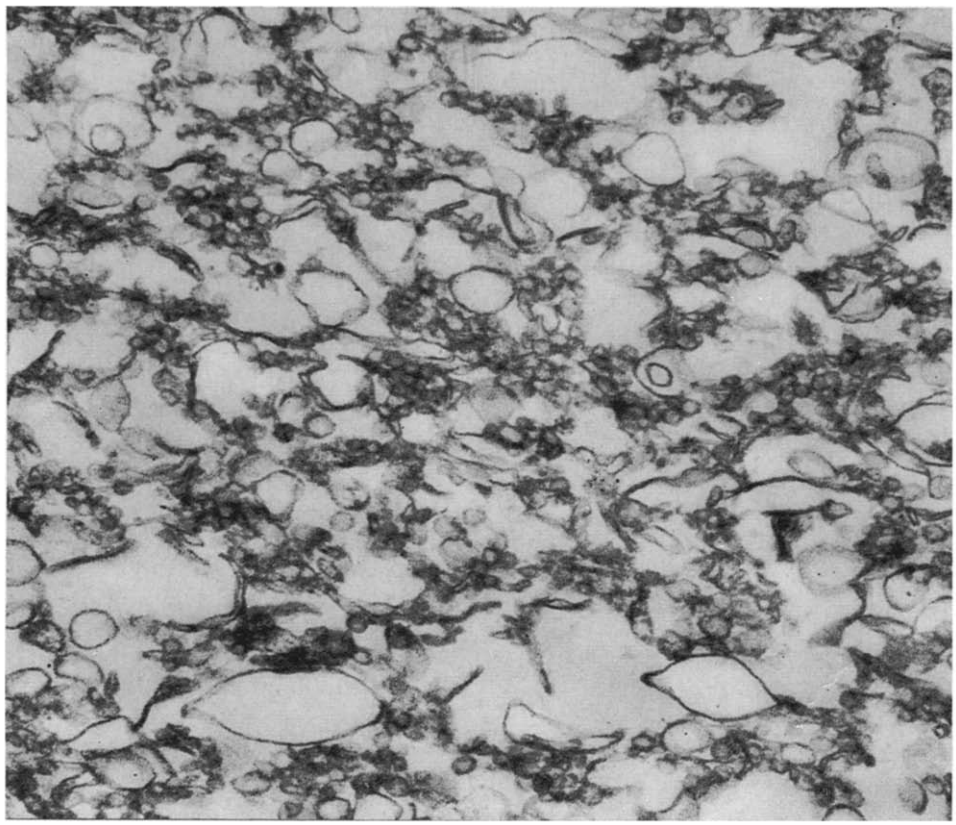


Fig. 5. Electronmicrograph of subfraction M_2 showing the presence of numerous synaptic vesicles and some contaminating membranous fragments. × 60 000.

various sizes probably originating from dendrites and other cellular processes and from intracellular membranes.

Table III shows that the microsomal gangliosides are about equally distributed between Mic-20 and Mic-100 with a slightly higher rel. spec. concn. in the membranes of the last fraction. The acetylcholinesterase follows a similar distribution.

Submitochondrial fractions (Table IB)

After the osmotic shock practically all the NANA of the mitochondrial fraction is recovered in subfractions M_1 and M_2 (Table II). M_1, which contains the bulk of the protein and particulate material, also has the larger proportion of NANA (*i.e.* 67.6%). However, because of the lower protein content, the spec. concn. ratio of NANA in subfraction M_2, which contains the synaptic vesicles and some membranes, is about twice that of M_1 (Fig. 3). The distribution of acetylcholinesterase in these submitochondrial fractions exactly parallels that of gangliosides (Table II).

Subfractions of M_1 (Table IC)

The subfractions of M_1 separated on a gradient show that gangliosides reach the highest concentrations (*i.e.* rel. spec. concn. 3.98 and 2.92) in subfractions M_1 0.9 and

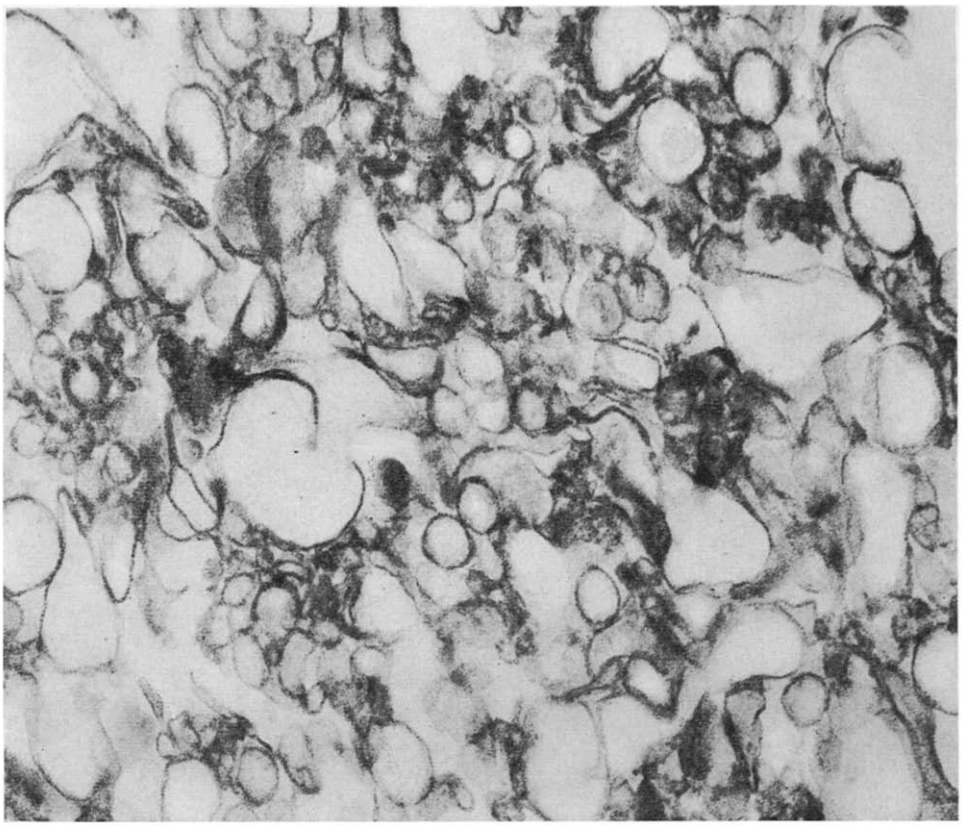


Fig. 6. Electronmicrograph of subfraction M_2B composed mainly of isolated membranes which contaminated subfraction M_2. These membranes have a high ganglioside concentration (see Table IV). × 60 000.

M_1 1.0 which contain synaptic membranes rich in acetylcholinesterase. Subfraction M_1 1.2, which contains a considerable amount of non-cholinergic synaptic membranes[15], shows little concentration of gangliosides (Table II and Fig. 3).

A detailed description of the morphological content of the subfractions of M_1, together with electron microscope illustrations, is given in ref. 2. Fig. 4 is an electron-micrograph of subfraction M_1 0.9, which has the highest concentration in gangliosides. The data of acetylcholinesterase and $(Na^+ + K^+)$-activated ATPase on these fractions as well as those on the binding of dimethyl-(+)-[^{14}C]tubocurarine[15] show that the distribution in subfractions of M_1 exactly follows that of gangliosides.

Subfractions of M_2 (Table ID)

The morphological content of subfraction M_2 from total rat brain was described by DE ROBERTIS *et al.*[12,13]. It contains the bulk of the synaptic vesicles separated from the nerve endings after the osmotic disruption, and in addition some contamination with membranous fragments (Fig. 5). The gradient of M_2 permits a more complete separation of the two components. Subfraction M_2A is composed almost exclusively of synaptic vesicles which have undergone a certain degree of clumping. Fig. 6 shows

TABLE IV

GANGLIOSIDES AND ACETYLCHOLINESTERASE IN SUBFRACTIONS OF M_2

	Protein (%)	*NANA*		*Acetylcholinesterase (rel. spec. activity)*
		%	*rel. spec. concn.*	
M_2A	56.0	35.4	0.65	0.68
M_2B	44.0	64.6	1.46	1.41
Recovery (%)	78	79		76

the morphological constitution of M_2B in which the membranous contaminant of M_2 is separated.

Table IV shows that the larger proportion and concentration of gangliosides is in the M_2B fraction with a rel. spec. concn. of 1.46. In the M_2A the rel. spec. concn. is only 0.65. In the same experiment the acetylcholinesterase has a striking similarity in distribution. In another experiment not indicated in Table IV the M_2A layer was completely separated from the supernatant lying above, and in this case the NANA content and the acetylcholinesterase activity of the synaptic vesicles were practically nil and could not be assayed. In the same experiment it was found that M_2B contained practically all the acetylcholinesterase activity (91.2%), the rest (8.8%) being in the supernatant above M_2A. A similar distribution was observed for gangliosides.

DISCUSSION

Because of the differences in species and methods of assays employed, and particularly in the cell fractionation procedures used, it is difficult to correlate the data presented here with those found in the literature. Our value of NANA (679 μg/g) in the total homogenate of the rat cortex agrees with those of SEMINARIO, HREN AND GOMEZ[10] and HESS AND ROLDE[17] in total rat brain but is considerably higher than those found by KOENIG *et al.*[22] in the same species. The per cent distribution of gangliosides in the primary fractions confirms most of the previous observations on the same material[10,22]. These glycolipids are concentrated in the crude mitochondrial fraction and particularly in the microsomal one, whereas there is practically nothing in the nuclear and soluble fractions. The finding by EICHBERG, WHITTAKER AND DAWSON[23] of 36% of NANA in the nuclear fraction can only be explained by contamination with other fractions due to the cell fractionation technique. (For a consideration of such technical problems see ref. 24.)

The separation of the microsomes into a heavy or Mic-20 fraction composed of small nerve endings, and a light or Mic-100 truly microsomal, shows that the gangliosides are somewhat more concentrated in the latter, which is composed mainly of membranous fragments probably arising from dendrites and other cellular processes and from intracellular membranes.

Previous investigations have shown that in the submitochondrial fractions gangliosides accompany the nerve endings[6,9,23], and particularly those of cholinergic nature[10,22]. In the work of SEMINARIO, HREN AND GOMEZ[10] it is particularly clear that these acid glycolipids are neither concentrated in myelin or free mitochondria nor in

the non-cholinergic endings of DE ROBERTIS *et al.*[11] which contain glutamic acid decarboxylase[25].

The study of the submitochondrial fractions after osmotic shock demonstrates that about 70% of the gangliosides is in the M_1 fraction. However, since the M_2 (vesicular fraction) contains little protein it has a higher rel. spec. concn. Similar findings by BURTON *et al.*[14] were interpreted as indicating that gangliosides are contained in synaptic vesicles. As a consequence, a role in the binding of acetylcholine and in the transport of this transmitter through the presynaptic membrane was postulated[14]. These interpretations are not supported by our finding that gangliosides of subfraction M_2 are present in a membranous contaminant (Fig. 6). Purification of M_2 on a gradient demonstrates that the synaptic vesicles are truly devoid of gangliosides. Similarly it is now proved that these vesicles do not contain acetylcholinesterase. The most interesting findings result from the separation on a gradient of the submitochondrial fraction M_1. This procedure allows the separation of two subfractions of synaptic membranes rich, and another subfraction poor, in acetylcholinesterase. Gangliosides reach the highest concentration in the cholinergic type of synaptic membranes. In these membranes (Na^+-K^+)-activated ATPase[21], adenyl cyclase and the particulate phosphodiesterase* are also concentrated. Since such membranes bind several cholinergic blocking agents it has been suggested that they contain the cholinergic receptor[26].

The ganglioside-rich nerve-ending fractions[10] not only contain acetylcholine but also serotonin[27], noradrenaline[28], dopamine[28] and histamine**. Thus the possibility exists that these glycolipids might be related to the receptor properties of the membrane toward some of the biogenic amines. Studying the effect of neuraminidase, on the sensitivity of muscle to serotonin, WOOLEY AND GOMMI[29] reached the conclusion that gangliosides may act as receptors for this amine. The strategic position of gangliosides in some of the synaptic membranes may be related to the presence of some specific amine receptor. The strict correlation with acetylcholinesterase and adenyl cyclase in the same membrane fractions is not incompatible with such an interpretation. The correlation with the Na^+- and K^+-activated ATPase in synaptic membranes[21] should not be disregarded and deserves further study; however, in the fractions studied[30] this relationship is not as strict as for acetylcholinesterase.

Although the highest concentration of gangliosides is reached in some synaptic membranes it is evident that other membranous components of certain neurons (*i.e.* dendritic or intracellular membranes) may also contain these complex glycolipids.

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** K. KATAOKA AND E. DE ROBERTIS, unpublished results.

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