Gangliosides of Bovine Adrenal Medulla*

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ABSTRACT: Gangliosides were isolated from bovine adrenal medulla and separated by thin-layer chromatography into five fractions. Total yield was 223 μg of lipid-bound sialic acid/g of fresh tissue, equivalent to about 840 μg of ganglioside. This amounted to 0.68 μmole of ganglioside, which is slightly over 50% the molar concentration in gray matter of brain. In terms of micromoles of ganglioside per gram of lipid, the adrenal medulla contained 75% as much as gray matter. Approximately 92% of the mixture consisted of two hematosides, AG_5 and AG_6, which contained *N*-glycolylneuraminic acid (NGNA) and *N*-acetylneuraminic acid (NANA), respectively. Both contained 1 unit each of glucose and galactose but no hexosamine. The remainder was a mixture of slow-migrating, brain-type gangliosides. One of these, AG_3, was found by gas–liquid partition analysis to contain glucose, galactose, galactosamine, and NANA in molar ratios of 1:1.93:0.96:1.98. One of the NANA units was removed by neuraminidase. Fraction AG_2 gave a similar though not identical analysis, except that approximately half the sialic acid was NGNA. It was not homogeneous, but the major component of AG_2 interestingly contained both NANA and NGNA in the same structure. Fraction AG_1 was a mixture but gave indication of another ganglioside containing both types of sialic acid.

Since their initial discovery as components of the central nervous system (Klenk, 1939) gangliosides have been studied primarily in relation to this organ, though in recent years their presence as minor lipids of several other tissues has been revealed (for review, see Svennerholm (1964) and Ledeen (1966)). The major species of brain possess more complex oligosaccharide structures than most gangliosides outside the CNS[1] and have the important property of containing hexosamine as well as sialic acid. The simpler hematoside[2] structure lacking hexosamine, which was isolated originally from erythrocytes (Yamakawa and Suzuki, 1951), has been found to constitute the major ganglioside of a number of extraneural organs (see Discussion).

It has been generally accepted for some years that the peripheral nervous system (PNS) contains little or no ganglioside. In actuality, there have been comparatively few studies in this area. Moreover, these have involved only sciatic nerve and a few other structures. Considering the near ubiquity of these substances it seemed worthwhile to examine other elements of the PNS, and we report here our findings for bovine adrenal medulla. This component of the autonomic nervous system has been found in the present study to contain an appreciable ganglioside concentration which, in terms of micromoles per gram, is about half that of brain gray matter. The molecular pattern, however, is quite different from brain in that the predominant species are two hematosides containing NANA and NGNA, respectively. Brain-type gangliosides are also present, and three such fractions were isolated along with the two hematosides. Carbohydrate studies of these fractions are described in this report.

Materials

Bovine adrenal glands obtained fresh from a local slaughterhouse were removed shortly after death and kept on ice until processing a few hours later. Merck reagent grade solvents were employed for extraction and chromatography and were redistilled for the latter purpose. Column chromatograms were prepared with "Unisil," an activated silicic acid (200–325 mesh) from the Clarkson Chemical Co., Inc., Williamsport, Pa. Silica gel G, E. Merck, was used for thin-layer chromatography. Neuraminidase (*Vibrio cholera*) was obtained from General Biochemicals, Chagrin Falls, Ohio. *N*-Acetyl- and *N*-glycolylneuraminic acids were from Sigma Chemical Co., St. Louis, Mo. Methyl nonadecanoate from Applied Science Laboratories, State College, Pa., was employed as an internal standard for gas–liquid partition chromatography. Sugar glycosides used as standards for gas–liquid partition chromatography were prepared in this laboratory. These included the methyl α- and β-D-glycopyranosides of glucose and galactose, and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside. Methyl ketoside methyl

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CNS, central nervous system; PNS, peripheral nervous system; NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid; MND, methyl nonadecanoate; TMS, trimethylsilyl.

[2] The term "hematoside" is employed here for the general structure, ceramide-lactose-sialic acid. The term "brain-type ganglioside" is used to denote those species which contain additional carbohydrates including hexosamine and which migrate on thin-layer chromatography in the same vicinity as the major species of brain; this does not, however, imply structural identity with the latter.

esters of NANA and NGNA were prepared according to the procedure of Kuhn *et al.* (1966), employing methanol with Dowex 50 (H^+) as catalyst.

Methods

Extraction and Analysis of Tissue. Adrenal glands were cut open and medulla was carefully dissected from cortex. In a typical run, 40 g of medulla was blended vigorously with 400 ml of chloroform–methyl alcohol (1:1) at room temperature. After filtering with a fritted-glass funnel of medium porosity, the residue was re-extracted with the same solvent by stirring under reflex for 2 hr. This mixture was cooled and filtered, and the filtrate was analyzed separately from that of the first extraction. The residue was dried and weighed to give an approximate measure of tissue protein.

The filtrates were brought to volume by addition of chloroform, and aliquots were removed for analyses. These included phosphorus (Chen *et al.*, 1956), protein (Lowry *et al.*, 1951), and sialic acid (Svennerholm, 1957; Miettinen and Takki-Luukainen, 1959). Other aliquots were treated with sufficient chloroform to adjust the chloroform–methyl alcohol ratio to 2:1 and then partitioned into two phases by addition of two-tenths volume of H_2O (Folch *et al.*, 1957). The lower phase was evaporated to dryness and weighed to give the fraction termed "C-M soluble (washed)," while aliquots of the original filtrate were taken to dryness to give the fraction termed "C-M soluble (unwashed)."

Total protein was determined by homogenizing weighed portions of fresh tissue with 0.3 M sucrose, dialyzing exhaustively (to remove catecholamines which interefere), and analyzing by the Lowry procedure. Sodium dodecyl sulfate was used to disperse most of the insoluble matter; a final light turbidity was removed by centrifugation following color development.

Sialic acid determination as described above suffered from the disadvantage that crude extracts were employed. More reliable estimates were obtained with enriched ganglioside fractions prepared as follows. Aliquots of the original filtrate from chloroform–methyl alcohol extraction, corresponding to about 1g of tissue, were evaporated to remove chloroform and transferred with warm methanol to prewashed dialysis bags. The tube was rinsed with a large volume of water into the bag. Following dialysis, the nondialyzable fraction was lyophilized to dryness and the residue treated with 6 ml of chloroform–methyl (1:2) plus 0.5 ml of 1 N KOH (aqueous) for 5 hr at 37°. Chloroform was removed by evaporation, water was added, and the sample was again dialyzed. The nondialyzable portion was lyophilized to dryness, and the residue was dispersed in chloroform and applied to a 5-g column of Unisil packed in chloroform; the sample tube was subsequently rinsed with each solvent as it was applied to the column. Most of the other lipids still present were eluted with 200 ml of chloroform followed by 500 ml of chloroform–methanol (83:17). Gangliosides along with some remaining lipids were then eluted with 800 ml of chloroform–methyl alcohol (1:1). Portions of this enriched ganglioside fraction were then analyzed for sialic acid by the resorcinol method.

Thin-Layer Chromatography. Plates were coated with a 250-μ layer of silica gel G and just before use were activated at 110° for 40 min. The solvent system, chloroform–methyl alcohol–2.5 N aqueous ammonia (60:40:9), was allowed to migrate within 1 in. of the top, and the plate was then dried 40 min in the atmosphere followed by another 20 min in a vacuum desiccator. It was then returned to the tank containing additional solvent for a second run. Best separation was achieved when two plates (20 × 20 cm) were run together in the same glass tank without paper lining.

A variation of the above procedure was the use of double-length plates (20 × 40 cm) with the same solvent. Three such plates were placed lengthwise in a circular tank 12 in. in diameter, without paper lining, which contained 400 ml of solvent. The latter ascended approximately four-fifths the length in a period of 4–6 hr. The plates were dried and returned for a second run.

For preparative purposes the silica gel was first extracted with organic solvents before spreading, in order to remove organic impurities. Extraction with chloroform–methyl alcohol (2:1) was followed by acetone, and the silica gel was then heated overnight at 120°.

For some purposes borate-impregnated plates were advantageous, and these were prepared according to the method of Kean (1966). The above solvent was employed. To determine proportions of individual ganglioside fractions, resorcinol assays were carried out directly on the silica gel scrapings without prior elution, according to the method of Suzuki (1964).

Separation of Individual Fractions. Preparative thin-layer chromatography was used to separate the ganglioside mixture and remove remaining lipids. The enriched ganglioside fraction prepared as above was first subjected to Folch partitioning (Folch *et al.*, 1957) with salt in the aqueous phase. Virtually all of the brain-type gangliosides, along with a small portion of the hematosides, went into the upper aqueous phase, but the bulk of hematosides remained in the lower phase. The latter two substances from the lower phase were separated by thin-layer chromatography using 20 × 20 cm plates. Rhodamine 6G (0.001% aqueous) was employed as the nondestructive spray and the bands were detected with ultraviolet light. The zones were scraped from the plate with a clean razor, leaving a small strip which was subsequently sprayed with resorcinol–HCl to confirm the presence of sialic acid. The two fractions were eluted by stirring the silica gel at room temperature with chloroform–methyl alcohol–water (50:50:15). The hematosides were then rechromatographed on silica gel–borate plates and extracted in the same manner. The products were dialyzed in the cold against distilled water and finally chromatographed on a small column of Unisil.

The brain-type gangliosides present in Folch upper phase were dialyzed to remove salt and then separated by thin-layer chromatography with double-length plates (*vide supra*). Forty plates were run with approximately 350 μg of sialic acid applied to each as a

streak along the short edge. Rhodamine 6G spray with ultraviolet light revealed six definite bands, the two fastest being the hematosides. The slower moving or brain-type fractions were extracted by packing the silica gel scrapings into a column as a chloroform slurry and eluting with chloroform–methyl alcohol mixtures. Two hundred milliliters of a 9:1 mixture removed rhodamine and 1 l. of a 1:1 mixture eluted most of the ganglioside. An additional small amount of ganglioside was obtained with chloroform–methyl alcohol (3:2) containing 5% water.

Determination of Sialic Acid Species. Both thin-layer chromatography and gas–liquid partition chromatography were employed for this purpose. Ganglioside samples containing 15–25 μg of sialic acid were dissolved in 0.5 ml of 0.03 N HCl (aqueous) and heated at 85° for 2 hr. This liberated most of the sialic acid. The mixture was partitioned with chloroform and the aqueous phase passed through a column of Dowex 2-X8 (acetate). After washing with water, sialic acid was eluted with 0.5 M sodium acetete. This solution was then passed through a column of Dowex 50W-X8 (H^+) to convert the salt into acid, and the resulting eluate was lyophilized to dryness. The residue was subjected to thin-layer chromatography with the system, *n*-propyl alcohol–1 N ammonia–water (6:2:1), according to the method of Granzer (1962). NGNA and NANA were detected with resorcinol spray and were usually well separated.

For the gas–liquid partition chromatography method, a ganglioside sample containing 15–20 μg of sialic acid was dissolved in 0.5 ml of 0.1 M HCl–methyl alcohol (anhydrous) and heated at 80° for 2.5 hr. This liberated sialic acid in the form of the methyl ketoside methyl ester. *N*-Acyl groups were not cleaved. The mixture was evaporated to dryness and placed in a vacuum desiccator with KOH for a few hours. The residue was then partitioned between water and chloroform and the former phase lyophilized. The resulting dry residue was converted to trimethylsilyl ether derivatives according to the procedure of Carter and Gaver (1967) and analyzed by gas–liquid partition chromatography with a 6-ft JXR column, coated 6% on Gas-Chrom Q (Applied Science Laboratories, Inc.). An F & M model 1609 gas chromatograph was employed with a hydrogen flame detector and helium carrier gas. Standards of NANA and NGNA were converted into the methyl ketoside esters (*vide supra*) for comparison. Peak areas were determined with a disc integrator.

Neuraminidase Treatment. Each ganglioside fraction, containing approximately 60 μg of sialic acid, was dissolved in 0.4 ml of 2 mM $CaCl_2$ (buffered at pH 5.3 with acetate) and treated with 0.1 ml of neuraminidase solution containing 50 units of enzyme. The mixture was covered with toluene and incubated at 37° for 2–3 days, during which time three or four additional 0.1-ml portions of neuraminidase were added. The toluene was removed by evaporation and the solution was dialyzed against a small volume of distilled water. The resulting dialysate was reduced to a small volume by lyophilization and the liberated sialic acid was identified by the thin-layer chromatography procedure described above; it was frequently necessary to purify the sialic acid first by the ion-exchange method described above. The nondialyzable portion containing glycolipid was also evaporated to dryness, extracted with chloroform–methanol (2:1), filtered, and analyzed by thin-layer chromatography. If this product was found to contain sialic acid (resistant to neuraminidase) the latter was cleaved by mild acid treatment and identified by thin-layer chromatography.

Carbohydrate Analyses. The chief method employed for both qualitative and quantitative determination of sugars was based on the gas–liquid partition chromatography method of Sweeley and Walker (1964). Approximately 0.1 μmole of each ganglioside fraction in a small tube with Teflon-lined screw cap was treated with 2.0 ml of 0.5 N HCl–methanol (anhydrous) and heated under nitrogen at 80° for 23 hr. The cooled sample was extracted four times with 3 ml of hexane and the combined hexane fraction was set aside for fatty acid analysis (*vide infra*). The methanolic solution was evaporated to dryness, desiccated under vacuum with KOH, and allowed to react for 1 day in the dark with 0.5 ml of methyl alcohol–acetic anhydride (3:1) plus a little silver acetate; this corresponds to procedure C of Sweeley and Walker (1964) and serves to reacetylate hexosamine and sialic acid. After filtration the solution was evaporated to dryness and the sugars were converted into TMS derivatives. The pyridine solvent used for the latter reaction was then replaced with a small volume (15–25 μl) of hexane for gas–liquid partition chromatography.

Prior to gas–liquid partition chromatography, measured amounts of methyl nonadecanoate were added to the TMS sugar mixture and to the fatty acid methyl esters in hexane; these served as internal standards for relating total fatty acid to carbohydrate composition. Both gas–liquid partition chromatography determinations were carried out with the same column employed for sialic acids (*vide supra*). Peak areas were quantified by means of a disc integrator. For the carbohydrate runs, temperature was raised in two discrete steps following emergence of the glucose and *N*-acetylhexosamine peaks, respectively. To determine relative detector response of the individual sugars, weighed samples of the following standards were analyzed by gas–liquid partition chromatography under similar conditions: the methyl α- and β-D-glycopyranosides of glucose and galactose, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside, methyl ketoside of NANA methyl ester, and methyl nonadecanoate. In some instances sugars were also identified by paper chromatography and analyzed by colorimetric methods according to procedures previously described (Ledeen and Salsman, 1965).

Results

Tissue Analysis. Table I summarizes the results of two sets of analyses. Using the resorcinol method for sialic acid determination, standard NGNA was found to yield 42% more color than the same weight of NANA. This fact, together with the observation that the two types were present in approximately equivalent amounts, enabled calculation of total lipid-bound sialic acid.

TABLE I: Composition of Bovine Adrenal Medulla.[a]

Trial	Water	Protein[b]	Phospholipid[c]	C-M Soluble[d] (unwashed)	C-M Soluble[e] (washed)	Lipid-Bound[f] Sialic Acid
1	778	132	33.9	61.7	42.3	0.228
2	781	139	33.2	60.7	39.7	0.218

[a] All values are expressed as milligrams per gram of fresh tissue. [b] Lowry determination. [c] Lipid phosphorus × 25. [d] Chloroform–methyl alcohol extract before washing with H_2O. [e] Previous fraction after washing with two-tenths volume of H_2O. [f] Resorcinol determination of enriched ganglioside fraction, corrected for difference in extinction coefficients between NANA and NGNA (see Methods).

The average value of 223 μg/g of fresh tissue would be equivalent to roughly 840 μg of ganglioside, using a value of 26.5% as the average composition of sialic acid. These results were obtained with the enriched ganglioside preparation from which most other lipids, catecholamines, etc., had been removed. It was somewhat surprising to find that resorcinol assay of the crude chloroform–methanol extract gave values only 10–15% higher.

In some instances the medulla tissue was extracted with cold acetone prior to chloroform–methyl alcohol treatment. Some ganglioside was removed by the acetone, but quantitative assay showed this to be only about 2% of the total, and the final yield of ganglioside was not affected. The second chloroform–methyl alcohol extract, obtained by stirring under reflux, was found to contain only 3–4% of the lipid-bound sialic acid. The principal extraction could be performed at either room temperature or reduced temperature.

Protein comprised over 60% of the dry weight of this tissue. Slightly over 6% of the tissue fresh weight (28% of dry weight) was extracted into chloroform–methyl alcohol (1:1) and approximately one-third of this partitioned into Folch upper phase. Lipids (lower phase) thus comprised close to 4% of fresh weight and almost 19% of dry weight. Phospholipids accounted for most of the lipid fraction, cholesterol was appreciable, but glycolipids were minor constituents. On Folch partitioning about half of the lipid-bound sialic acid was found to enter the upper phase in the absence of salt, but this was calculated to constitute only 3–4% of total upper-phase material. Protein in the upper phase was estimated to be slightly less, so that the bulk of the upper-phase solid was unidentified material. Catecholamines and other low molecular weight substances were undoubtedly part of this fraction.

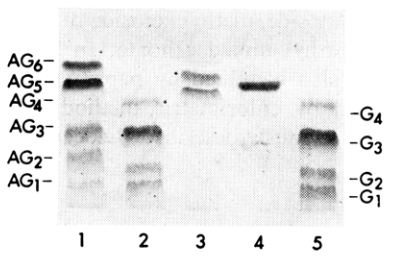

FIGURE 1: Thin-layer chromatogram of gangliosides of beef adrenal medulla and brain. On silica gel G; solvent, chloroform–methyl alcohol–2.5 N ammonia (60:40:9); resorcinol spray, all visible bands are purple. (1) Adrenal medulla, Folch upper phase (with salt); (2 and 5) brain ganglioside mixture; (3) adrenal medulla, enriched ganglioside fraction prepared from Folch lower phase; (4) Tay-Sachs ganglioside. For explanation of symbols, see footnote 3.

Lowry determination indicated that 3.5% of chloroform–methyl alcohol soluble material was protein, of which 72% remained in the lower phase after partitioning. The latter conforms to the operational definition of proteolipid protein, which thus constitutes 0.15% of tissue fresh weight and 0.70% of dry weight.

Ganglioside Pattern. Ganglioside thin-layer chromatography patterns for adrenal medulla and brain are compared in Figure 1. Several differences are evident. The former has large quantities of two fast-moving species, AG_5 and AG_6.[3] Both of these were subsequently shown to be hematosides (*vide infra*). A portion of these partitioned into Folch upper phase, even in the presence of salt, but the majority remained in the lower phase. Adrenal medulla also has a small amount of the slower migrating, brain-type gangliosides, all of which went into the upper phase. Three such fractions were clearly discernible on the chromatogram while a fourth (AG_4) was very faint. Although these migrated in the same region as brain species they were not exactly parallel; this suggested differences in carbohydrate structures which were subsequently found by analysis.

The relative proportions of these fractions were determined by the thin-layer chromatography method of Suzuki (1964), employing aliquots of both upper and lower phases from the enriched ganglioside preparation. The slow-moving brain-type species were analyzed as a group and found to comprise 7.8% of the total, based on sialic acid. AG_5 and AG_6 were 47 and 45%, respectively.

Ganglioside Separation. Preparative thin-layer chromatography was found to be the only satisfactory

[3] Symbols used to designate these fractions are based on thin-layer chromatography migratory rates, the prefix "A" denoting adrenal. The symbols for brain gangliosides are those of Korey and Gonatas (1963), which are correlated with the symbols of Svennerholm (1963a) as follows: $G_4 = G_{M1}$; $G_3 = G_{D1a}$; $G_2 = G_{D1b}$; $G_1 = G_{T1}$.

method for resolving the mixture into individual ganglioside fractions. The brain-type species were separated best on double-length plates, yielding three major fractions and one minor; the latter (AG_4) could not be obtained in sufficient quantity for analytical studies. In isolating the two hematosides, samples of high purity were obtained by sequential use of plates coated with silica gel G and borate-treated silica gel. Sphingomyelin, one of the major contaminants, overlapped with AG_5 on the former plate but moved well ahead on the latter due to retardation of the glycolipid by borate. Somewhat less purity was achieved for some of the slower fractions, as indicated by analysis (*vide infra*). The yields were estimated at 30–40% for AG_1, AG_2, and AG_3, and slightly higher for the hematosides. These separated fractions are shown in Figure 2.

Carbohydrate Analysis. The gas–liquid partition chromatography method employed, essentially that of Sweeley and Walker (1964), was based on their demonstration that the methyl glycosides of ganglioside sugars are separated as TMS derivatives on nonpolar columns. Several quantitative runs with Tay-Sachs ganglioside and normal brain species of known composition served to confirm the reliability of the method with respect to glucose, galactose, and galactosamine; sialic acid, while generally accurate, sometimes gave values which were significantly lower than those obtained with resorcinol. Qualitative identification of sialic acid type was not possible in these runs since the original acyl group belonging to the amide function was removed during methanolysis. Following the procedure as outlined it was possible to determine all the ganglioside carbohydrates as well as fatty acid content. Long-chain bases were not included in this procedure, although in principle they could have been. Gas–liquid partition chromatography of the TMS derivative of *N*-acetylsphingosine showed that these bases had very long retention times and emerged from the JXR column after NANA.

For quantification it was necessary to determine the relative response of each carbohydrate to the flame detector, using known quantities of standards. Each standard was run as a mixture with methyl nonadecanoate (MND), and the relative detector response (*DR*) was computed with respect to this internal standard

$$DR = \frac{(\text{area of glycoside})(\text{mg of MND})}{(\text{mg of glycoside})(\text{area of MND})}$$

The anomers of glucose and galactose were determined separately and a weighted average (*DR'*) was calculated based on the actual proportions of α and β anomers produced by methanolysis of glycolipid. Galactose gives rise to a third peak (presumably methyl galactofuranoside) for which a standard was not available, but since this was the smallest of the three peaks its omission from the calculation of *DR'* introduced little error. The latter peak was, of course, included in area summation of galactose. For *N*-acetylgalactosamine and NANA methyl ester only one methyl glycoside standard was available, but in each case this corresponded to the major anomer formed by methanolysis. *N*-Acetylgalactosamine gave rise to a few minor peaks, as reported by Sweeley and Walker (1964), and the two most important of these emerged from the column before the major peak. These were probably furanosides rather than the β-pyranoside. NANA had two minor peaks, one before and one after the major one, and both were necessary for quantification.

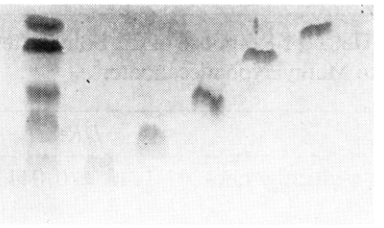

FIGURE 2: Thin-layer chromatogram of isolated ganglioside fractions from beef adrenal medulla. Conditions same as Figure 1. (M) Original ganglioside mixture from Folch upper phase.

Results are summarized in Table II. Glucose was found to have a larger detector response than galactose, the difference being due to the α anomers. Penick and McCluer (1966) also found a greater response for glucose, though their ratio for the α anomers of the two sugars was slightly less than ours. These workers reported the same detector response for the two anomers of glucose, whereas our results indicated a difference of about 7%.

The quantity of each sugar was computed with the formula

$$\mu\text{moles of sugar} = \frac{(\text{area of sugar})(\mu\text{g of MND})}{(DR')(\text{area of MND})(\text{mol wt})}$$

where (μg of MND) is the weight of internal standard added to the glycoside mixture. The term "(area of sugar)" is the integrated sum of all peaks for a given carbohydrate. For galactosamine and sialic acid the *DR* values for the single isomers were employed in place of *DR'*. A similar formula was employed for calculating micromoles of total fatty acid, with *DR'* set equal to one. Methyl esters of the fatty acids, which were extracted with hexane following methanolysis, were determined by gas–liquid partition chromatography separately from the carbohydrates. The individual acids were assumed to have the same detector response as methyl nonadecanoate for purposes of calculation, even though this was not strictly true; the error arising from this assumption, however, would be small. Over-all fatty acid pattern indicated an average composition of approximately C_{20}, and a molecular weight of 326 corresponding to methyl arachidate was therefore used to compute micromoles of fatty acid. The use of an internal standard enabled correlation of total fatty acid with the various sugar components.

Results are given in Table III. Galactosamine was the only hexosamine detected in the brain-type gangliosides, and glucose and galactose were the only hexoses. These identifications were con-

TABLE II: Detector Response of Carbohydrate Standards Relative to Methyl Nonadecanoate.

	DR[a]	*DR'*[b]
Methyl α-D-glucopyranoside	1.46 ± 0.044	1.43
Methyl β-D-glucopyranoside	1.36 ± 0.039	
Methyl α-D-galactopyranoside	1.21 ± 0.032	1.25
Methyl β-D-galactopyranoside	1.36 ± 0.033	
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	0.82 ± 0.037	
NANA-methyl ketoside methyl ester	0.77 ± 0.016	

[a] Detector response plus and minus standard deviation; based on four or more determinations. [b] Detector response computed as a weighted average, based on ratios determined from gas–liquid partition chromatography; glucose, $\alpha/\beta = 2.62$; galactose, $\alpha/\beta = 2.70$.

firmed by paper chromatography. AG_5 and AG_6 were shown by both methods to contain glucose and galactose but no hexosamine. The latter observation was confirmed by colorimetric assay. These two each had the composition of hematoside, since both gas–liquid partition chromatography and resorcinol assay indicated one sialic acid attached to ceramide–lactoside. Their chief difference was the type of sialic acid, AG_5 containing NGNA and AG_6 NANA (Table IV).

AG_2 and AG_3 analyzed as disialo species and also migrated on thin-layer chromatography in the same general region as the disialogangliosides of brain

TABLE III: Composition of Adrenal Medulla Gangliosides.[a]

Component	Ganglioside				
	AG_1	AG_2	AG_3	AG_5	AG_6
Glucose	1	1	1	1	1
Galactose	1.70	1.52	1.93	0.97	1.11
Galactosamine	0.58	0.83	0.96		
Sialic acid (gas–liquid partition chromatography)	1.68	1.76	1.98	0.86	0.95
Sialic acid (resorcinol)	2.41	2.07	2.23	0.97	1.29
Fatty acid	4.27	0.92	1.36	1.28	1.07

[a] Expressed as molar ratios relative to glucose as one. All values except "sialic acid (resorcinol)" were determined by gas–liquid partition chromatography.

TABLE IV: Sialic Acid Composition of Ganglioside Fractions, Determined by Gas–Liquid Partition Chromatography. Effect of Neuraminidase.

Fraction	Weight %		Liberated by Neuraminidase[a]
	NANA	NGNA	
AG_1	47.8	52.2	NANA + NGNA
AG_2	57.6	42.2	NANA
AG_3	85.4	14.6	NANA
AG_5	3.4	96.6	NGNA
AG_6	100.0	0.0	NANA

[a] The major products only, determined qualitatively by thin-layer chromatography.

(Figure 1). AG_3 further resembled the latter in having two units of galactose and one galactosamine per unit of glucose, but AG_2 departed somewhat from these integral ratios. Subsequent studies with neuraminidase (*vide infra*) revealed that all of the slow-moving fractions contained a second minor ganglioside in addition to the major component, and this fact may account for the analytical ambiguity. Fraction AG_1 gave variable results for sialic acid depending on the method, but appeared closer to a disialo composition; its thin-layer chromatography migration, however, was close to that of brain trisialoganglioside. The unusually high fatty acid composition indicated that this fraction was very likely contaminated with one or more other lipids.

Determination of Sialic Acid Species. The sialic acid compositions of the various fractions, determined by gas–liquid partition chromatography, are given in Table IV. This method is particularly suitable for both qualitative and quantitative study of sialic acids. However, we subsequently discovered from the use of model compounds that the methanolysis conditions employed would have removed any *O*-acetyl groups present. Sialic acids of this type have been isolated from other sources (Gottschalk, 1960). We therefore repeated the study using milder conditions (0.03 M HCl–anhydrous methanol, 80°, 35 min), which model compounds had shown left about 60% of *O*-acetyl groups intact. These conditions were still sufficient to liberate a significant proportion of bound sialic acid. Gas–liquid partition chromatography analysis of the products showed that AG_5 and AG_6 contained little if any *O*-acetylated sialic acids. The other ganglioside fractions were not studied in this manner.

Neuraminidase Treatment. AG_5 and AG_6 lost all their sialic acid on reaction with neuraminidase, giving a product in each case which migrated parallel to ceramide–lactoside on thin-layer chromatography. Some chromatograms showed this product as one band and others as two very close ones; both gangliosides behaved similarly in this respect.

The three brain-type fractions lost only part of their sialic acid with neuraminidase. Thin-layer chromatography of the resulting glycolipid products is shown

diagrammatically in Figure 3. Each gave rise to a major and a minor product, both still containing sialic acid. This was taken as evidence for the presence in each fraction of a minor ganglioside. The major ganglioside product from AG_3 migrated parallel to AG_4, while the minor product coincided with AG_3 itself and was therefore apparently unchanged by the enzyme. The sialic acid liberated from AG_3 was almost entirely NANA (Table IV), with only a trace of NGNA. The major ganglioside product, isolated by thin-layer chromatography, was found to contain only NANA. It is probable that the 14–15% of NGNA present in original fraction AG_3 (Table IV) arose from the minor component which only became visible as the faint, slower moving band after neuraminidase.

The sialic acid liberated from AG_2 by the enzyme was primarily NANA, with only a small amount (estimated 5–10%) of NGNA. In this case the major ganglioside product was slower moving, migrating parallel to original AG_3; this substance was isolated by thin-layer chromatography and found to contain NGNA only. These obervations showed that this major product could not be the same as AG_3, despite its parallel migration on thin-layer chromatography. In addition, the analytical data required this to be a monosialo species. An important conclusion concerning the major ganglioside in the original fraction AG_2 is that it must contain both NANA and NGNA in its oligosaccharide chain. The minor neuraminidase product from AG_2 migrated parallel to AG_4 and was found to contain NANA. It evidently originated from a minor ganglioside component of fraction AG_2 and probably accounts for the fact that NANA and NGNA were not precisely equimolar in this fraction.

The ganglioside products from AG_1 were quite similar in thin-layer chromatography behavior to those from AG_2, but the liberated sialic acid included both NANA and NGNA in approximately equal amounts. One possible interpretation is that fraction AG_1 contains a major component which is a trisialo species with one NANA and two NGNA units. The minor component would then contain relatively more NANA. However, other possibilities have not been ruled out. Much of the difficulty is undoubtedly due to the greater inhomogeneity of this fraction.

Discussion

The prevailing belief that gangliosides are generally lacking in the PNS requires some revision in view of the present study, which demonstrates that at least one structure within this network contains a relatively high concentration of these substances. In an earlier study of sciatic nerve, Folch *et al.* (1958) reported a very low level of ganglioside: 0.011% weight of fresh tissue. This corresponded to about 0.028% when corrected for the presence of connective tissue, and this value may be compared to 0.084% found here for adrenal medulla. More recently sciatic nerve was claimed (Lowden and Wolfe, 1964) to have no ganglioside, and a similar result was obtained by these workers for the sympathetic chain. The lipids of intradural spinal roots have been

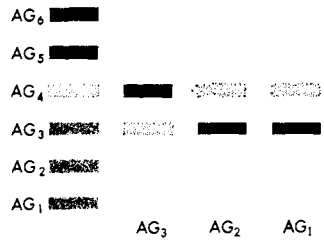

FIGURE 3: Diagrammatic representation of glycolipid products from neuraminidase treatment of ganglioside fractions. Thin-layer chromatography as in Figure 1. All visible bands contained sialic acid; the sialic acids remaining in fractions AG_1, AG_2, and AG_3 were resistant to neuraminidase.

studied (O'Brien *et al.*, 1967) and also reported to contain no ganglioside. It is possible, therefore, that the adrenal medulla is atypical in this regard, though the regions surveyed to date are too few to permit such a conclusion. In addition it might well prove beneficial to reexamine the above PNS structures with techniques which have proved suitable for hematosides as well as brain-type gangliosides, since these often behave differently during isolation.[4]

Lipids of the adrenal medulla were previously studied in regard to subcellular distribution (Douglas *et al.*, 1966; Blaschko *et al.*, 1967). These studies dealt with cholesterol and several phospholipids, but little attention was accorded the glycolipids. While the present work has shown this tissue to contain relatively high levels of gangliosides in comparison to other tissues outside the CNS (*vide infra*), they nevertheless comprise only 2% of total lipid. Other glycolipids of unknown structure were detected during the course of this work, but these also were minor constituents. Glycolipid as a whole undoubtedly comprises only a few per cent of total lipid.

Over 90% of the gangliosides in the adrenal medulla were the hematoside type, and the presence of both NANA- and NGNA-containing species in the same tissue was somewhat unusual. Each of these accounted for about 46% of total lipid-bound sialic acid. Carbohydrate analysis showed that these two gangliosides differed only in the type of sialic acid, and this was verified by the formation of ceramide–lactoside from both with neuraminidase. It was surprising to find that this small structural difference caused such a relatively large difference in thin-layer chromatography behavior; the NGNA-containing hematoside (AG_5) migrated not only behind the NANA-containing hematoside (AG_6) but also behind Tay-Sachs ganglioside, which contains an additional sugar (Figure 1). This effect occurred with the chloroform–methyl alcohol–ammonia system described previously, but when propyl alcohol–water (7:3) was employed, AG_5 migrated ahead of Tay-Sachs ganglioside. The reason for this difference is not known.

[4] We have found, for example, that approximately 85% of adrenal medulla gangliosides remain in the lower phase on Folch partitioning with aqueous salt solutions. We have also observed differences in elution pattern with DEAE-cellulose chromatography, the method used by O'Brien *et al.* (1967).

TABLE V: Lipid-Bound Sialic Acid Concentration in Adrenal Medulla and Brain.[a]

	Adrenal Medulla	Gray Matter	White Matter
mg of sialic acid/g fresh weight	0.223	0.785	0.232
mg of sialic acid/g dry weight	1.01	4.43	0.869
mg of sialic acid/g of lipid	5.44	13.6	1.59
μmoles of ganglioside/g fresh weight	0.680	1.27	0.383
μmoles of ganglioside/g dry weight	3.09	7.17	1.41
μmoles of ganglioside/g of lipid	16.6	22.0	2.56

[a] Expressed as milligrams of sialic acid and micromoles of ganglioside. Values for brain sialic acid are from Ledeen *et al.* (1968). Water contents of gray and white matter were taken as 82.3 and 73.3%, respectively (same reference). Lipid percentages of dry weight for gray matter (32.7%) and white matter (54.9%) are those of Norton *et al.* (1966). For brain, the average composition is close to two sialic acids per mole of ganglioside (Suzuki, 1964). For adrenal medulla, we used the composition found here: 92.2% monosialo and 7.8% disialo species (it would introduce only a slight error in the total figure if AG_1 is actually a trisialo).

Whereas brain itself contains only minute amounts of hematosides (Suzuki, 1964), several extraneural tissues contain these substances as the major ganglioside type. This has been demonstrated for spleen (Svennerholm, 1963b), liver (Eeg-Olofsson *et al.*, 1966), placenta (Svennerholm, 1965), erythrocytes (Yamakawa and Suzuki, 1951; Klenk and Padberg, 1962), and lung (Gallai-Hatchard and Gray, 1966). The major gangliosides of lens (Feldman *et al.*, 1966) and intestine (Vance *et al.*, 1966) have slightly more complex carbohydrate structures but resemble the hematosides in lacking hexosamine. Gangliosides which contain hexosamine, such as the major species of brain, are therefore a rarity outside the CNS, and it is apparent that most of the adrenal medulla gangliosides conform to the extraneural pattern.

This study has demonstrated, however, that the adrenal medulla does contain a small portion (7–8%) of ganglioside that resembles brain structures in containing hexosamine. Three such fractions have been isolated in varying degrees of purity and shown to contain two or more sialic acids, an additional feature characteristic of most brain gangliosides. Another compound (AG_4), possibly a monosialo, was detected on thin-layer chromatography but was not isolated in sufficient quantity for characterization. Each of the three isolated fractions was shown by neuraminidase treatment to contain at least two gangliosides. However, the major components of AG_2 and AG_3 appeared to constitute 80% or more of their respective fractions, so that some structural conclusions in these cases were possible.

The major ganglioside of fraction AG_3 was shown to contain two NANA units, one of which was removed by neuraminidase. Its carbohydrate composition is the same as G_3 as brain (Svennerholm's G_{D1b}), but it moved slightly ahead of the latter on thin-layer chromatography and may therefore contain a different carbohydrate arrangement. The major ganglioside of fraction AG_2 also appears to have two sialic acids per molecule. A particularly interesting feature of this substance is the presence of both NANA and NGNA in the same structure. To our knowledge this is the first such ganglioside to be reported. The data suggested that fraction AG_1 might also contain a ganglioside with both types of sialic acid, though the apparent contamination of this fraction with other lipid made interpretation difficult.

It is of some interest to compare the total level of gangliosides in the adrenal medulla with those of other tissues. Using the values in Table I and an average sialic acid content of 26.5% (computed from the composition of the known species), the weight of total ganglioside is 840 μg/g fresh weight and 3.81 mg/g dry weight. These values are significantly higher than those obtained for placenta by Svennerholm (1965): 155 μg/g fresh weight and 1.2 mg/g dry weight. The latter tissue was claimed by Svennerholm to contain more ganglioside than all other organs studied (kidney, liver, lung, lymph nodes, and muscle) except spleen, which contained 50% more, and of course brain. The adrenal medulla thus contains a larger ganglioside concentration than any of the extraneural organs studied to date.

Table V presents a comparison with brain levels, calculated in a number of different ways. Expressed as milligrams of sialic acid per gram fresh weight, adrenal medulla has approximately the same content as white matter and slightly over one-fourth that of gray. However, when these values are converted into micromoles of ganglioside, adrenal has slightly over 50% as much as gray matter and about 1.8 times that of white. The adrenal content approaches that of gray matter most closely when expressed as micromoles of ganglioside per gram of lipid, in which terms it is 75% of the latter. The level of lipid-bound sialic acid in brain (Ledeen *et al.*, 1968) was converted into micromoles of ganglioside on the basis of an average disialo composition, while for adrenal the conversion was based on the presence of 92.2% monosialo species and the remainder disialo, according to the results of this study. Values used for the lipid percentages of brain were those of Norton *et al.* (1966).

It is difficult to know which of these various modes of expression has the greater significance in reference to physiological function, but it seems likely that molar rather than weight concentrations are more meaningful. This still leaves open the question of whether it is moles of sialic acid or moles of ganglioside to be considered. There is now firm evidence that most gangliosides of

the CNS occur in the neuronal membrane (Derry and Wolfe, 1967), though a small portion is probably associated with glia (Ledeen *et al.*, 1968). Subcellular studies with brain have indicated high concentrations of gangliosides at the synapse and/or adjoining membrane (Lapetina *et al.*, 1967), and there is evidence which suggests that their role in such membranes is involved in some manner with the cation transport mechanism (McIlwain, 1963). In the adrenal medulla, depolarization of the chromaffin cell by acetylcholine is accompanied by an influx of ions, which leads to release of catecholamines from the chromaffin granules (Douglas and Poisner, 1962). By analogy with the CNS it is conceivable that the gangliosides of this tissue might also serve in some capacity related to ion transport, but there is no evidence as yet to support this notion. It would be of considerable interest to know whether the brain-type gangliosides of the adrenal reside in specific membranes distinctive from those containing the hematosides, as this might provide a clue to possible differences in function of the two classes of ganglioside. Subcellular studies currently in progress may help to elucidate this question.

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