In connection with the tryptic sensitivity our results agree with the many observations made in this field and especially those of Rigopoulou et al. (1970) and Noe and Bauer (1971). Tager and Steiner (1973) provided direct chemical evidence indicating the reason of the tryptic sensitivity of mammalian proglucagons.

From the various studies on the conversion of the prohormones proparathyroid (Habener et al., 1973), large gastrin (Gregory and Tracy, 1972), β-lipotrophin (Cretien and Li, 1967), proinsulin (Steiner et al., 1972), and now proglucagon to their respective hormones, a general pattern of conversion emerges in which tryptic-like enzymes play a fundamental role.

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Structures of Gangliosides from Bovine Adrenal Medulla†

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ABSTRACT: Five gangliosides, accounting for over 95% of the total ganglioside fraction, were isolated from bovine adrenal medulla by preparative thin-layer chromatography and the carbohydrate structures determined by a combination of periodate oxidation and permethylation techniques. Partially methylated alditol acetates were generated from the neutral sugars of the fully methylated glycolipids and identified by gas–liquid chromatography. Substitution on *N*-acetylgalactosamine was determined by methanolysis of the permethylated ganglioside, acetylation of the products, and identification of the resulting substituted methyl glycosides by GLC. Periodate oxidation followed by borohydride reduction confirmed some of the linkages and demonstrated the absence of (2–8) linkages between sialic acid units. Mass spectrometry of the permethylated gangliosides gave information on sugar sequence at the nonreducing end.

Gangliosides were previously shown to occur at appreciable levels in bovine adrenal medulla (Ledeen et al., 1968a), the molar concentration amounting to approximately half that of bovine gray matter on a fresh weight basis. These gangliosides, although obtained from a component of the autonomic nervous system, appeared to have more in common with gangliosides of extraneural organs than of brain. Thus, over 90% of the mixture was hematoside, and the sialic acid was evenly divided between NANAc[1] and NANGly.[2] Lipophilic components were also of the extraneural

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[1] Abbreviations used are: NANAc, *N*-acetylneuraminic acid; NANGly, *N*-glycolylneuraminic acid; Gal, galactose; Glu, glucose; GalNAc, *N*-acetylgalactosamine; Cer, ceramide; NANAc-7, NANAc with two terminal carbons removed; NANGly-7, NANGly with two terminal carbons removed.

[2] Brain has little hematoside and very little NANGly (cf. Yu and Ledeen, 1970; Ledeen and Yu, 1973).

type in that fatty acids were of variable chain length (C_{14}–C_{24}) and C_{20}-sphinogosine was absent (Ledeen and Salsman, 1970).

Molar ratios of carbohydrate constituents were previously determined for several of these individual fractions (Ledeen et al., 1968a). We now report a more detailed study of oligosaccharide structures for three monosialo- and two disialogangliosides which represent over 95% of total ganglioside in this gland. Substitution positions on the neutral sugars were determined by generation of partially methylated alditol acetates (Björndal et al., 1967, 1970), while periodate-borohydride treatment gave supplementary data. Substitution on *N*-acetylgalactosamine was established by a recently developed procedure utilizing methyl glycoside derivatives of this sugar (Kundu et al., 1975). Information on carbohydrate sequence was obtained by mass spectrometric analysis of the permethylated gangliosides.

Materials and Methods

Isolation of Gangliosides. Bovine adrenal medullae were processed as previously described (Ledeen et al., 1968a) and the gangliosides purified with the aid of DEAE-Sephadex and Unisil chromatography (Ledeen et al., 1973). Individual fractions were isolated by preparative thin-layer chromatography (TLC) (Ledeen et al., 1968a). Gangliosides were eluted from the silica gel G scrapings by stirring an hour with chloroform–methanol–water (50:40:15, v/v); addition of Dowex 50W-X8 resin (200–400 mesh, Na^+ form) to the stirred mixture (approximately 4 ml of resin/g of silica gel) increased recovery of ganglioside, possibly through removal of calcium from the medium.

Neuraminidase. Small samples (0.2–0.5 μmol) of ganglioside were dissolved in 0.3–0.5 ml of dilute acetic acid (pH ~5) and treated with 0.05 ml of neuraminidase solution (0.2–0.4 unit) prepared in the same medium. Sigma type VI enzyme from *Clostridium perfringens* was employed. The solution was overlayered with toluene and incubated several hours at 37°. Additional portions of enzyme were added over 24–48 hr. The toluene was removed by evaporation with nitrogen and the aqueous mixture treated with 20 volumes of chloroform–methanol (2:1, v/v). The resulting precipitate was removed by filtration and the filtrate evaporated to dryness. The residue from the filtrate was dissolved in methanol–chloroform–water (60:30:8, v/v) and passed through a DEAE-Sephadex A-25 column as previously described (Ledeen et al., 1973). Those gangliosides which retained one unit of sialic acid after neuraminidase (AG_2, AG_3, and AG_4) were recovered in the second fraction, eluted from DEAE-Sephadex with solvent containing sodium acetate, while those which lost all their sialic acid (AG_5 and AG_6) gave neutral glycosphingolipid which was eluted from DEAE-Sephadex in the first fraction (containing no salt).

Permethylation Studies. Individual gangliosides and their neuraminidase products were subjected to permethylation as previously described (Kundu et al., 1975). We employed the reagent developed by Hakomori (1964) (viz., methyl iodide plus dimethylsulfinyl carbanion) which effected rapid and complete methylation of all free hydroxyl and –NH amide groups. The products were purified by preparative thin-layer chromatography (TLC) before subsequent reaction.

Partially methylated alditol acetates were produced from the various neutral sugars by a procedure based on that of Björndal et al. (1970); 50–100 μg of purified methylated ganglioside were heated 3 hr at 100° in 0.5 ml of 88% formic acid; after evaporation of solvent the residue was heated an additional 18 hr in 1 ml of 0.3 *N* hydrochloric acid (aqueous) at 100°. Solvent was again removed by evaporation. The residue was dissolved in 2 ml of water and passed through a small column (1 ml of resin) of Dowex 50W (H^+) to remove hexosamine, followed by washing with 6–7 ml of water. The solvent was concentrated to approximately 1 ml and treated with 15 mg of sodium borohydride. After 4 hr at room temperature, excess borohydride was destroyed with a few drops of glacial acetic acid, bringing the solution to pH 5. The mixture was evaporated to dryness and the residue evaporated four times with 1–2 ml of methanol containing a drop of acetic acid. The final residue was thoroughly dried, dissolved in 0.5 ml of acetic anhydride–pyridine (1:1, v/v), and heated 25 min at 100°. Solvent was removed with a nitrogen stream followed by vacuum desiccation. The partially methylated alditol acetates were extracted from the dried residue with chloroform, employing mild sonication, and purified by chromatography on a 1-g Unisil column. The mixture was applied to the latter in benzene; the column was eluted with 30 ml of benzene to remove impurities and then with 30 ml of chloroform to elute the alditol acetates. For gas–liquid chromatography (GLC) analysis the mixture was dissolved in a small volume of chloroform and injected onto an OV-225 column operated isothermally at 180°.

Substitution on *N*-acetylgalactosamine was determined by a recently described procedure (Kundu et al., 1975) utilizing methyl glycosides of the substituted hexosamine. These were generated by subjecting permethylated glycolipid to acid-catalyzed methanolysis followed by acetylation of the resulting free hydroxyl. Identification of these products was accomplished by GLC on OV-1 and OV-225. The method was applied to approximately 20 μg of glycolipid.

Periodate–Borohydride Treatment. Samples of 0.15–0.50 μmol of adrenal medulla gangliosides and various brain ganglioside standards were dissolved in 1.5 ml of chloroform–methanol (1:2) and treated with 0.2 ml of 0.8 *M* sodium metaperiodate (aqueous). After the mixture stood 2–3 days at room temperature excess periodate was reduced with ethylene glycol; the mixture was dialyzed and evaporated to dryness. The residue was dissolved in 2 ml of solution composed of 20 mg of sodium borohydride, 1.5 ml of methanol, and 0.5 ml of 0.001 *N* sodium hydroxide. After standing 2 hr in ice the mixture was held at room temperature for another 2 hr and then brought to pH 5–6 with acetic acid. Dialysis followed by evaporation gave a residue which was subjected to GLC analysis following either vigorous or mild methanolysis (see below).

Vigorous Methanolysis. Half of the above sample was heated in 2 ml of 0.75 *N* methanolic HCl at 80° for 24 hr. After evaporation to dryness a portion of the residue was analyzed for threitol and erythritol by acetylation for 19 hr at room temperature in 0.5 ml of acetic anhydride–pyridine (1:1); the solvent was evaporated and the products (in hexane) were subjected to GLC analysis isothermally at 115° on an OV-1 column. Another portion was analyzed for undestroyed glucose, galactose, and galactosamine by acetylation with 0.5 ml of acetic anhydride–methanol (1:3) containing a pinch of silver acetate, according to the method of Vance and Sweeley (1967); this resulted in acetylation of amino but not hydroxyl groups. After filtration and evaporation to dryness the residue was converted to Me_3Si derivatives by the method of Carter and Gaver (1967) and chro-

Table I: Structures of Adrenal Medulla Gangliosides.[a]

Symbol[b]	Corresponding Brain Ganglioside[c]	
AG_6	G_6	NANAc (2–3) Gal (1–4) Glu (1–1) Cer
AG_5		NANGly (2–3) Gal (1–4) Glu (1–1) Cer
AG_4	G_4	Gal (1–3) GalNAc (1–4) Gal (1–4) Glu (1–1) Cer (3, 2) NANAc
AG_3	G_3	Gal (1–3) GalNAc (1–4) Gal (1–4) Glu (1–1) Cer (3, 1, 2) (3, 1, 2) NANAc NANAc
AG_2		Gal (1–3) GalNAc (1–4) Gal (1–4) Glu (1–1) Cer (3, 1, 2) (3, 2) NANAc NGNAc

[a] Glycosidic bond configurations were not determined in this study. In brain gangliosides all such bonds were shown to have the β configuration (for review see Ledeen and Yu, 1973) except NANAc whose ketoside configuration is α (Yu and Ledeen, 1969). Since these structures are analogous to those of brain gangliosides it is likely their glycosidic configurations are the same. [b] These were the symbols previously assigned to adrenal medulla gangliosides (Ledeen et al., 1968a). [c] Nomenclature system of Korey and Gonatas (1963). The corresponding Svennerholm (1963) symbols are: $G_6 = G_{M_3}$; $G_4 = G_{M_1}$; $G_3 = G_{D_{1a}}$. Svennerholm symbols have not been assigned to the other structures.

matographed isothermally at 155° on an OV-1 column.

Mild Methanolysis. Part of the original product from the above periodate-borohydride treatment was subjected to mild methanolysis in 0.05 *N* methanolic HCl as previously described (Yu and Ledeen, 1970). This was designed to reveal sialic acid units that had not been destroyed by periodate. Terminal sialic acid groups are converted by the periodate-borohydride treatment to products with two carbons less (NANAc-7 and NANGly-7) and these were also identified by GLC following mild methanolysis; standards for such identification were previously synthesized (Yu and Ledeen, 1969).

Small portions of the original ganglioside were also subjected to mild methanolysis to obtain information on sugar sequence within the oligosaccharide chain. The samples were heated 2 hr and in some cases 3.5 hr at 80° in 0.05 *N* methanolic HCl, cooled, and extracted three times with hexane. The methanolic solution was evaporated to dryness with a stream of nitrogen, converted to the Me_3Si derivatives and chromatographed on OV-1. Glucose, galactose, and galactosamine were analyzed isothermally at 155° while sialic acid was determined at 205° with phenyl *N*-acetyl-α-D-glucosaminide as internal standard (Yu and Ledeen, 1970).

Gas–Liquid Chromatography. GLC was carried out with a Hewlett-Packard F&M Model 402 instrument equipped with flame ionization detector. U-shaped glass columns, 6 ft × 4 mm, were employed isothermally with two types of packing: OV-225, 3% on Supelcoport (100–120 mesh); and OV-1, 3% on Chromosorb W HP (100–120 mesh). Both packings were obtained from Supelco, Inc., Bellefonte, Pa. Helium carrier gas was employed at a flow rate of approximately 70 ml/min. Peak areas were integrated with an Infotronics CRS-101 electronic integrator.

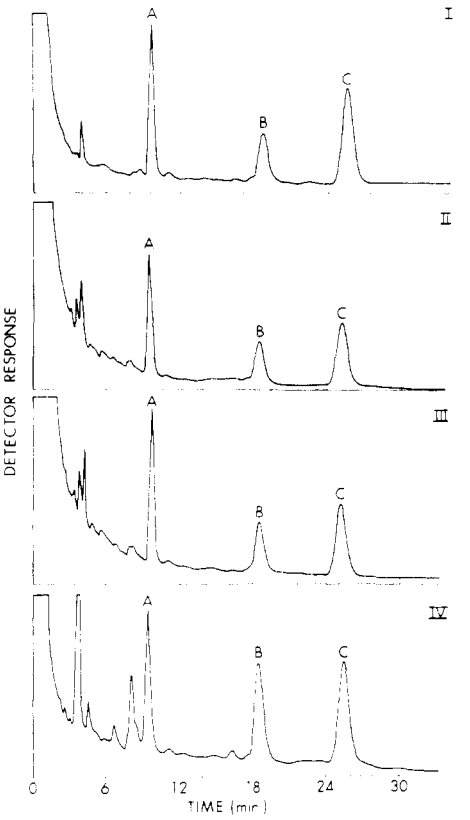

FIGURE 1: Gas–liquid chromatograms of partially methylated alditol acetates from monosialogangliosides. I, $G_4(G_{M1})$ from brain; II, AG_4 from adrenal medulla; III, major neuraminidase product of AG_3; IV, major neuraminidase product of AG_2. Peak identities: A, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; B, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol; C, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol. Chromatograms were obtained on OV-225 operated isothermally at 150°.

Mass Spectrometry. Mass spectra were recorded on an RMU-6E (Hitachi-Perkin Elmer, Norwalk, Conn.) single focussing medium resolution instrument. Operating conditions were: filament current 100 μA, electron energy 70 eV, acceleration voltage 6 kV, ion source temperature, 230°. The permethylated glycolipids were deposited in a quartz receptacle and injected by direct inlet. The assistance of Dr. Paul Gallop and Mr. Ed Henson is gratefully acknowledged.

Results and Discussion

Isolation. The five ganglioside fractions obtained by preparative TLC appeared homogeneous on rechromatography, but two of these (AG_2 and AG_3) were subsequently found to be mixtures. The main evidence for this was formation of two gangliosides by exhaustive neuraminidase treatment, as described in the earlier study (Ledeen et al., 1968a). However, one product predominated in each case, and this comprised an estimated 80% or more of the fraction. Structural studies were limited to the major components of the two original fractions, which are designated herein as AG_2 and AG_3, respectively. AG_4, AG_5, and AG_6 appeared sufficiently homogeneous to be considered single molecular species. Carbohydrate ratios and sialic acid types were confirmed as corresponding to those previously determined (Ledeen et al., 1968a). The AG_1 fraction reported in the earlier study (Ledeen et al., 1968a) was not obtained here in sufficient yield for detailed structural analysis.

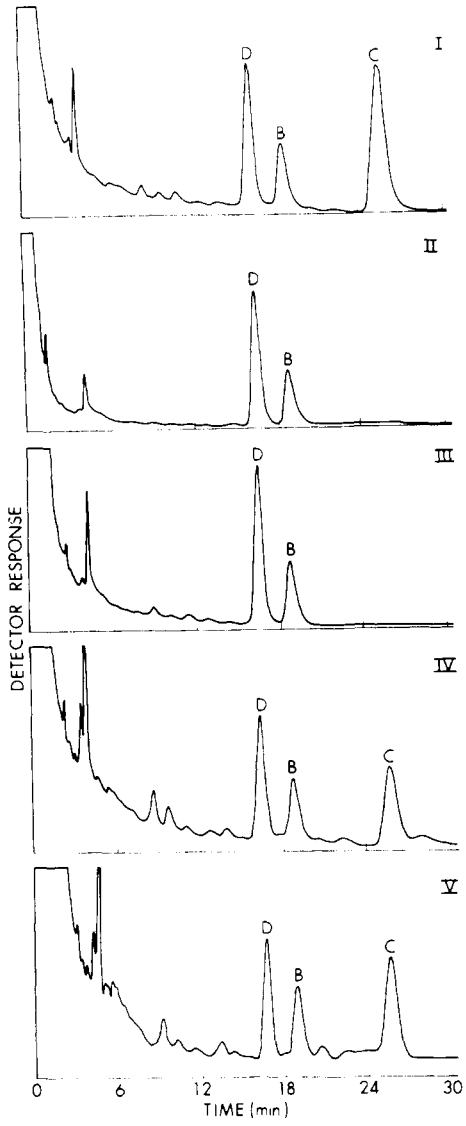

FIGURE 2: Gas–liquid chromatograms of partially methylated alditol acetates from mono- and disialogangliosides. I, $G_3(G_{Dla})$ from brain; II, AG_6; III, AG_5; IV, AG_3; V, AG_2. Peak identities: D, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; B and C same as in Figure 1. Chromatograms were obtained on OV-225 operated isothermally at 150°.

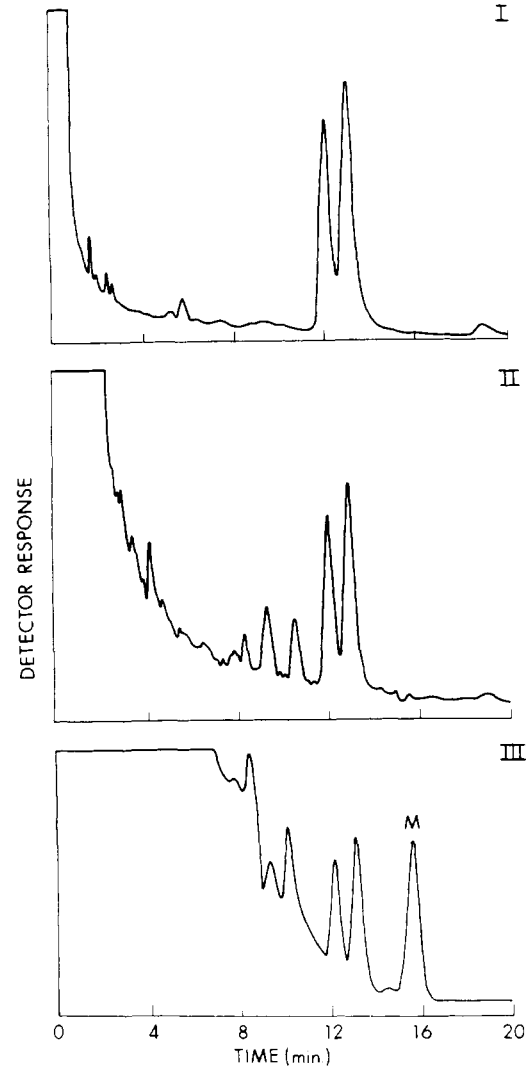

FIGURE 3: Gas–liquid chromatograms of galactosamine methyl glycosides. I, synthetic methyl glycosides of 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactose (cf. Kundu et al., 1975); II, products from methanolysis of permethylated asialo G_4 of brain (cf. Kundu et al., 1975); III, products from methanolysis of permethylated AG_4. Chromatograms were obtained on OV-225 operated isothermally at 180°. M is mannitol hexaacetate.

Structure Determinations: General. Results of structural studies are summarized in Table I. Glycosidic substitution sites were determined in the case of neutral sugars by the alditol acetate procedure in which each sugar gives rise to a single product that is characterized by GLC on OV-225. This column packing was found in our laboratory to be somewhat more effective than ECNSS-M, the one originally described (Björndal et al., 1967) for this purpose. Standards for comparison were prepared from brain gangliosides and other glycolipids of known structure. Chromatograms of these products are depicted in Figures 1 and 2. The small early peaks between the solvent front and peak A are of unknown identity and may represent impurities from the solvents or silica gel; the prominence of these artifacts in Figure 1-IV could reflect the smaller size of this sample.

Hexosamine did not give satisfactory yields of partially methylated alditol acetates by this procedure, but the substitution position on this sugar was elucidated by a recently described procedure utilizing methyl glycosides (Kundu et al., 1975). A portion of the permethylated ganglioside was subjected to methanolysis under relatively mild conditions and the resulting methyl glycosides were acetylated. The products were identified by GLC on OV-225 and confirmed on OV-1 columns. The three galactosamine-containing gangliosides (AG_2, AG_3, AG_4) generated the same two hexosamine peaks (along with methyl glycosides of the neutral sugars, which were eluted close to the solvent front); this is illustrated for AG_4 in Figure 3. These peaks corresponded to the two methyl glycosides of 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactose, prepared synthetically (Kundu et al., 1975), and by similar treatment of the major monosialoganglioside of brain (Figure 3). The 4,6-di-*O*-methyl derivative was readily distinguished from the other possible derivatives of *N*-acetylgalactosamine (Kundu et al., 1975) and the method thus established substitution on the 3-hydroxyl of this sugar for AG_2, AG_3, and AG_4.

Some glycosidic sites were confirmed by periodate–borohydride treatment. It was observed that all five adrenal medulla gangliosides so treated gave erythritol as one product, identified by GLC of the tetra-*O*-acetyl derivative; this

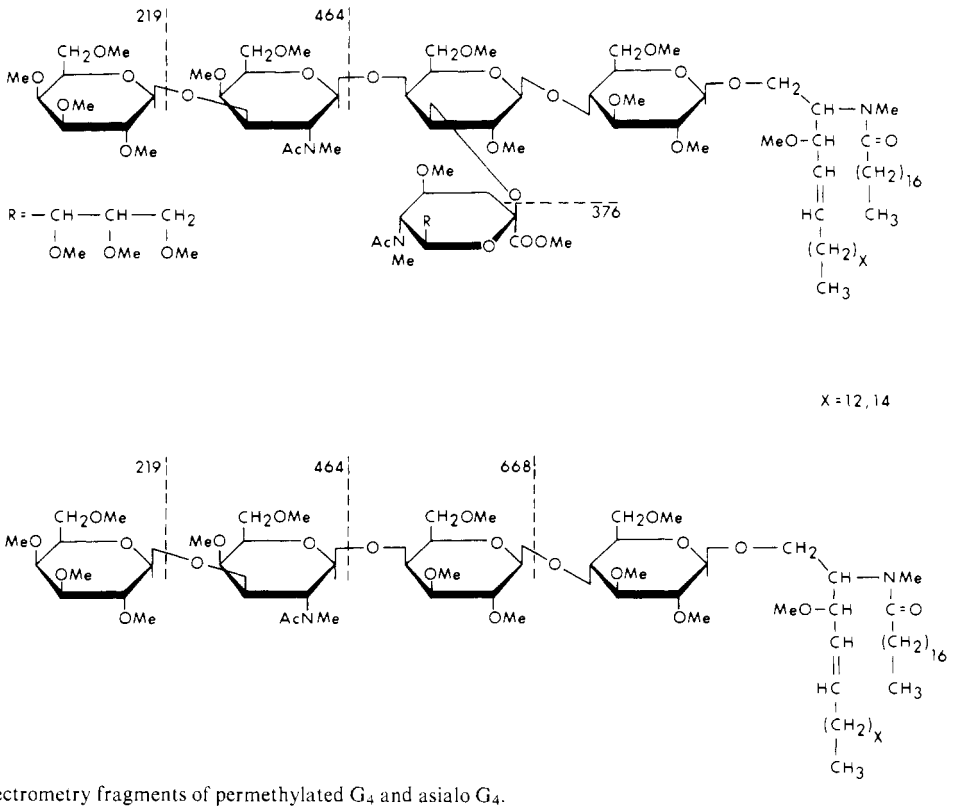

FIGURE 4: Mass spectrometry fragments of permethylated G_4 and asialo G_4.

indicated substitution on the 4-hydroxyl of glucose (Kuhn and Wiegandt, 1963). Periodate treatment also resulted in destruction of all sialic acid residues in the five gangliosides and formation of derivatives containing two carbons less: NANAc-7 and NANGly-7. The latter were identified by GLC comparison with standards previously synthesized (Yu and Ledeen, 1969). This showed the absence of structures containing two sialic acids linked together by a (2–8) bond, such as are found in some of the brain gangliosides (Kuhn and Wiegandt, 1964; Klenk et al., 1967; Ledeen et al., 1968b). One brain disialoganglioside known to contain a NANAc(2–8)NANAc linkage (G_2)[3] was subjected to the same treatment and gave GLC peaks corresponding to both NANAc and NANAc-7. The findings reported herein do not of course preclude the existence of such linkages among the minor adrenal medulla gangliosides not included in this study.

The permethylation sequence leading to alditol acetates and the periodate–borohydride treatment do not provide information on sugar sequence, with the exception of the terminal sugar(s) at the nonreducing end(s) of the oligosaccharide chain. Mass spectrometry of permethylated glycolipids was recently shown (Ledeen et al., 1974) to produce characteristic fragments which indicate part of the sequence, in particular the location of hexosamine units. The relevant fragments for brain ganglioside G_4 and asialo G_4 are shown in Figure 4. The sialic acid fragment at *m/e* 376 was detected intermittently for this compound provided a quartz sample container was used, but it could not be detected at all with glass containers. Glass apparently catalyzes destruction of the heat-liable sialic acid moieties, as observed previously with some of the lower molecular weight gangliosides (Ledeen et al., 1974). For those gangliosides containing NANGly the corresponding peak would be at *m/e* 406. The small peak at *m/e* 668 corresponding to the triglycosyl fragment of asialo G_4 was not present for the ganglioside.

Additional data on sugar sequence were obtained by partial methanolysis of the ganglioside under mild conditions, followed by GLC assay of the liberated monosaccharide methyl glycosides (as Me_3Si ethers). Using brain G_4 as a standard, 2 hr of heating at 80° in 0.05 *N* methanolic HCl gave the following molar ratios: Gal/Glu 16:2, NANAc/Glu 15.7, GalNAc/Gal 0.59, NANAc/GalNAc 1.7. The latter two values are reasonably close to the theoretical ratios (0.5 and 1.0, respectively), indicating that these three sugars are liberated to about the same extent under the mild conditions employed. Glucose alone among the sugars was liberated in much lower yield, indicating probable attachment to ceramide. The relative stability of the glucose-ceramide glycosidic bond has been noted previously (Sweeley and Vance, 1967; Zanetta et al., 1972). Ratios obtained by similar treatment of adrenal medulla gangliosides indicated glucose as the unit attached to ceramide in these species also. These data together with the results from mass spectrometry indicate the probable sequence Gal-GalNAc-Gal-Glu-Cer for the oligosaccharide backbone of these hexosamine-containing gangliosides.

Structures of AG_5 and AG_6. These two gangliosides occur in approximately equal concentrations in the bovine gland and together comprise over 90% of the total. Their hematoside character was demonstrated by absence of hexosamine and the presence of equimolar amounts of glucose, galactose, and sialic acid. The only detectable difference between the two was the nature of the sialic acid moiety, this being NANGly for the slower migrating AG_5 and NANAc for AG_6. Both substances lost their sialic acid on neuraminidase treatment to give lactosylceramide. Glycosidic substitutions were revealed by the nature of the alditol acetates

[3] This corresponds to G_{D1b} in the Svennerhotm (1963) system.

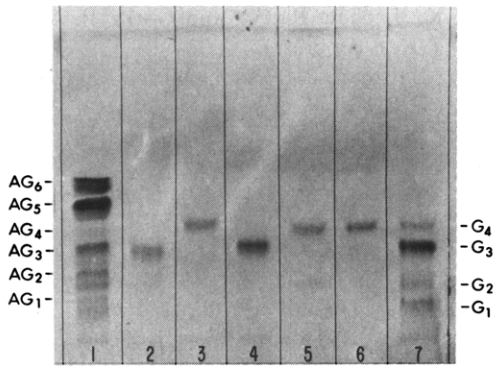

FIGURE 5: Thin-layer chromatogram of some adrenal medulla gangliosides and their neuraminidase products. 1, mixture of gangliosides from bovine adrenal medulla; 2, major neuraminidase product of AG_2; 3, major neuraminidase product of AG_3; 4, AG_3; 5, AG_4; 6, G_4 = major monosialoganglioside of brain; 7, mixture of bovine brain gangliosides. A precoated plate of silica gel G (Analtech), 250 μm thick, was activated 45 min at 110° before spotting; two ascending runs were carried out with chloroform–methanol–2.5 *N* NH_4OH (60:40:9, v/v). Resorcinol spray; all bands were purple. Symbols on left were those we previously assigned to adrenal medulla gangliosides (Ledeen et al., 1968a); those on the right are the Korey and Gonatas (1963) designations for brain gangliosides.

generated from the permethylated glycolipids (Figure 2): formation of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol indicated substitution for galactose and glucose at the 3- and 4-hydroxyl groups, respectively. This was confirmed by detection of erythritol and unchanged galactose after periodate–borohydride treatment. These results establish hematoside structures for AG_5 and AG_6 identical with those found in other tissues (for review see Ledeen and Yu, 1973).

Structure of AG_4. This ganglioside was barely detectable in the original mixture (Figure 5) and required many preparative TLC runs to obtain a few hundred micrograms. It comigrated with brain G_4 (Figure 5) and was similar to the latter in carbohydrate composition and in its resistance to neuraminidase. Formation of methylated alditol acetates from the neutral sugars gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,3,4,5-tetra-*O*-acetyl-2-6-di-*O*-methylgalactitol, and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol (Figure 1) which were identical with the products obtained from the major monosialoganglioside of brain (G_4).. Methanolysis of the permethylated ganglioside gave hexosamine products corresponding to substitution on the 3-hydroxyl of *N*-acetylgalactosamine (Figure 3). Mass spectrometry of the permethylated ganglioside gave ion fragments at *m/e* 219 and 464, indicating the same terminal disaccharide unit as brain G_4 (Figure 4). The oligosaccharide structure of AG_4 thus appears identical with that of the major monosialoganglioside of brain.

Structure of AG_3. GLC analysis revealed the major component of this fraction to be a disialoganglioside containing 2 NANAc units. Neuraminidase treatment gave a major product that comigrated on TLC with AG_4 and also a minor ganglioside that migrated the same as original AG_3. Structural studies were carried out with the major product only, and the results were virtually identical with those obtained with AG_4. These included the methylated alditol acetates derived from the three neutral sugars, methyl glycosides of methylated *N*-acetylgalactosamine, and mass spectrometry fragmentation patterns. Periodate–borohydride treatment of original AG_3 gave erythritol (substitution on 4-hydroxyl of glucose), NANAc-7, and unchanged galactose and *N*-acetylgalactosamine. Conversion of all NANAc to NANAc-7 by this reaction showed that the two sialic acids are attached to different positions on the oligosaccharide chain rather than to each other. The position of the neuraminidase-labile NANAc was indicated by comparison of the partially methylated alditol acetates of AG_3 and AG_4 (Figures 1 and 2): both gangliosides gave rise to 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, but the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol product of AG_4 was replaced by 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol in AG_3. This indicated the second NANAc to be located on the 3-hydroxyl of terminal galactose. Mass spectrometry of the neuraminidase product of AG_3 (following permethylation) gave the same pattern as AG_4. Mild methanolysis of the same neuraminidase product gave sugar ratios very similar to those obtained for G_4 (see above): Gal/Glu 16.4, NANAc/Glu 15.5, GalNAc/Gal 0.45, NANAc/GalNAc 2.0. Glucose thus shows evidence of being the sugar linked to ceramide and the carbohydrate sequence is apparently the same as for G_4 and AG_4. The oligosaccharide structure of AG_3 is thus identical with that of G_3, the major disialoganglioside of brain.[5]

Structure of AG_2. This ganglioside had essentially the same carbohydrate composition as AG_3, with one NANAc unit replaced by NANGly. Methylated alditol acetates of AG_2 and its neuraminidase product were identical with those from AG_3 and its neuraminidase product, respectively (Figures 1 and 2), while the galactosamine products resulting from methanolysis of permethylated AG_2 were identical with those from AG_3 and AG_4. Periodate–borohydride treatment resulted in formation of erythritol, indicating substitution on the 4-hydroxyl of glucose. In addition both sialic acids were converted to their lower homologs, NANAc-7 and NANGly-7, respectively. The basic structure of AG_2 is thus the same as AG_3 except for sialic acid type. The position of NANGly was established by neuraminidase, which left this sialic acid as part of the resulting monosialoganglioside product while removing NANAc. The former is thus attached to the 3-hydroxyl of internal galactose. Mass spectrometry of the permethylated neuraminidase product gave prominent peaks at *m/e* 219 and 464, indicating the same sequence as G_4 and AG_4 (Figure 4). This was supported by the results of mild methanolysis which suggested glucose attachment to ceramide.

Conclusion

Hexosamine-containing gangliosides comprise less than 10% of the total in bovine adrenal medulla, and the present study has demonstrated that three of these have carbohydrate structures closely analogous to the major gangliosides of brain. Using techniques effective at the microgram level, AG_4 has been shown to have an oligosaccharide chain identical with G_4, the major monosialoganglioside of brain. AG_3 was similarly shown to have the same carbohydrate structure as G_3, the major disialoganglioside of brain. AG_2 possesses the same tetraglycosyl backbone as AG_3 but has NANAc and NANGly attached to different galactoses. AG_6, the major NANAc-containing hematoside, is present

[4] This corresponds to G_{M1} in the Svennerholm (1963) system.

[5] This corresponds to G_{D1a} in the Svennerholm (1963) system.

as a minor ganglioside of brain (Svennerholm, 1963) while AG_5, the major NANGly counterpart, has not yet been detected in brain. Additional minor gangliosides are present in the adrenal medulla, as indicated by unidentified TLC bands and the presence of a small amount of glucosamine detected in the hexosamine fraction. The five species elucidated here are estimated to comprise well over 95% of total ganglioside in this tissue. While this study has pointed up many similarities to brain gangliosides the overall pattern, particularly in regard to hematoside predominance, sialic acid type and lipophilic composition (Ledeen and Salsman, 1970), indicate a closer relationship to gangliosides of extraneural organs (for review, see Ledeen and Yu, 1973).

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Chloride Flux in Bilayer Membranes: The Electrically Silent Chloride Flux in Semispherical Bilayers†

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ABSTRACT: High resistance semispherical bilayer membranes of areas as large as 0.3 cm^2 were formed from a decane solution of synthetic diphytanoylphosphatidylcholine. These bilayers had a specific resistance of about 10^9 Ω cm^2 and a specific capacitance of 0.38 μF cm^{-2} at 20° in 0.1 *M* KCl. Under these conditions, chloride permeability was 6.8×10^{-8} cm/sec. This electrically silent ^{36}Cl flux was found to be about 10^3-fold larger than the chloride current calculated from the electrical parameters of the system. The chloride flux in the bilayer was independent of the applied electrical field and was unaltered by addition of reducing agents to the ambient aqueous solutions. It was, however, substantially reduced when NO_3^- was substituted for Cl^- on the side of the bilayer initially free of ^{36}Cl, or if I^- was added to the aqueous phases in the concentration range of 0.001-0.1 *M*. These results strongly suggest that the electrically silent flux of ^{36}Cl is primarily a carrier mediated diffusion process in which phosphatidylcholine acts as the carrier species.

Most current concepts of biological membrane structure are based on the premise that the phospholipid component is present in bilayer form and as such constitutes a barrier matrix for the organization of protein and carbohydrate components. Because of this central position of the bilayer in membrane structure, extensive studies of the molecular organization and physical properties of bilayers have been carried out in many laboratories (Tien and Diana, 1968; Rothfield and Finkelstein, 1968; Mueller and Rudin, 1969; Thompson and Henn, 1970; Bangham, 1972). Considerable attention has been directed toward the permeabilities of bilayers to cations, but relatively little attention has been given to anion permeabilities.

Pagano and Thompson studied chloride permeability in spherical bilayers of large area by both electrical and isoto-

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