extractability of phylloerythrin from bile was tested by adding fresh ox bile to equal volumes of buffer solutions and shaking with ether. In solutions below pH 5 almost all the phylloerythrin was extracted into the ether. Above pH 7·5 less than 5% of the phylloerythrin was present in the ether phase. Between pH 5 and 7·5 intermediate values were obtained. This behaviour could be imitated by adding phylloerythrin (dissolved in a small volume of dilute hydrochloric acid) to solutions of sodium tauroglycocholate, and similar results were also obtained with strong soap solutions. On the other hand, with phosphate buffer solutions of pH 7·5 distribution of phylloerythrin was strongly in favour of the ether phase. As soaps and bile salts in aqueous solution are colloidal electrolytes (Roepke & Mason, 1940) it is concluded that phylloerythrin is present in bile in the free state and is probably maintained in solution by adsorption on the colloidal micelles.

Results from the study of bile suggest that in blood phylloerythrin is dispersed on colloidal micelles such as the bile salts and the plasma proteins. In support of this hypothesis is the observation (Rimington & Quin, 1934) that in sheep affected with geeldikkop phylloerythrin was present entirely in the plasma, only traces being found in the well-washed corpuscles.

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Studies on the Structure of Brain Ganglioside

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The brain gangliosides are macromolecular glycolipids first described by Klenk (1942). They are known to contain neuraminic acid, sphingosine, fatty acids, hexose and hexosamine, although little is known of the manner in which these constituents are combined. The present study reports detailed structural evidence obtained by stepwise hydrolytic cleavage of a highly purified preparation of brain ganglioside leading to the isolation and characterization of a constituent glucocerebroside, and the formulation of the structure of the repeating unit.

METHODS

Nitrogen. The digestion (Gunning, 1889; Arnold & Wedemeyer, 1892) was continued for 45 min. after the solution had become colourless. Water (14 ml.) and one drop of 1% gum ghatti were then added. After 6 min., 8 ml. of Nessler's reagent (Koch & McMeekin, 1924) was added to successive tubes at 45 sec. intervals, the colour allowed to develop for 9 min. and then read at 435 mμ (M. Lees, unpublished work).

Phosphorus. Phosphorus was determined spectrophotometrically (Sperry, 1942).

Neuraminic acid. The humin reaction (Folch, Arsove & Meath, 1951), the direct Ehrlich's reaction (Odin & Werner, 1952) and Bial's orcinol reaction (Bohm, Dauber & Baumeister, 1954) were employed. The Bial's reaction was found to be the most reliable.

Reducing sugar. The methods of Hagedorn & Jensen (1922) and of Hanes (1929) were adapted. Results were expressed as equivalents of galactose or glucose.

Hexose. Hexose was estimated by a modified orcinol–H_2SO_4 method (Sorensen & Haugaard, 1933) and expressed as equivalents of glucose or galactose. Since the absolute values differed slightly for the two sugars, identification of the hexose by paper chromatography indicated which curve was to be used. When glucose and galactose were together in a single fraction, the hexose was expressed as equivalents of galactose, since galactose was always in two- to three-fold excess.

Determination of hexosamine. The following modifications of the Elson & Morgan (1934) reaction were employed: 1 ml. of acetylacetone (6% in 2N-carbonate buffer) was added to a 2 ml. aqueous sample. The covered tube was heated at 100° for 20 min. Colour was developed with Ehrlich's reagent (2·6%) for 45 min. and read at 530 mμ. The standard curve, based on a purified sample of galactosamine hydrochloride, was verified for each determination. This modified method was suitable for estimation of 5–40 μg. of galactosamine (maximum error 3%). Maximum values were obtained after the subfractions were hydrolysed with N-HCl in a sealed tube for 16 hr.

Identification of the hexosamine. The method of Stoffyn & Jeanloz (1954), Gardell, Heijkenskjold & Roch-Norlund (1950) was used. The reaction with ninhydrin was carried out in a sealed capillary tube. The solvent system was modified (see chromatography methods). The amino sugar fractions used were first freed from neutral sugars and other contaminants by chromatography on a column of Dowex 50.

Amino nitrogen. Primary amino nitrogen was determined by the method of Van Slyke (1929).

Paper chromatography. Whatman no. 1 or no. 54 paper was irrigated downwards with *n*-butanol–ethanol–water (4:2:1, by vol.) for 12–72 hr., depending on the separation desired. Silver nitrate was used to develop reducing substances (Trevelyan, Procter & Harrison, 1950), ninhydrin for free amino groups and Ehrlich's reagent for neuraminic acid.

Ion-exchange fractionation. Dowex 50 resin (Boas, 1953) was used to separate neutral from amino sugars. Water was used to elute the neutral sugars, and 2N-HCl to elute the amino sugars.

Dialysis. Washed cellophan was used. In exhaustive dialysis, the outside water was changed at 12 hr. intervals until no solids appeared in the diffusate.

Preparation of brain ganglioside. Hot methanol extraction of whole ox cerebral hemispheres, freed from investing meninges and blood vessels, was employed, followed by precipitation in the cold, and partition between the two phases formed by $CHCl_3$–methanol (2:1, v/v) and water (12·2%, by vol.) (Folch, Meath & Bogoch, 1956; Bogoch, 1956). The upper-phase material, freed from solvents and dialysed, was crude brain ganglioside (P, 0·8%; yield, 2 g./kg. of fresh cerebral hemispheres).

Crude ganglioside was purified by repeated treatment in $CHCl_3$–methanol (2:1, v/v) and water (6·6%, by vol.). (Final yield, 1 g./kg. of whole cerebral hemispheres.) The ash content was reduced from 11% to less than 1·1% by repeated acidification and dialysis in the cold (Folch *et al.* 1951). The final preparation was completely water-soluble, free of phospholipids (P, 0·07%) and of dialysable contaminants. It contained N 2·9%, hexose 24% (expressed as galactose), neuraminic acid 30·3% and free amino N 0·5%. The preparation was homogeneous by electrophoresis, and over 95% homogeneous in the ultracentrifuge. Minimum molecular weight, 250 000 (Bogoch, 1956).

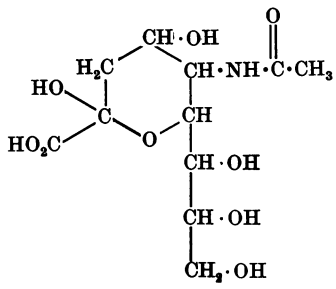
Note on nomenclature

The original and classical name, brain ganglioside, proposed by Klenk (1942) is adhered to in the present study. The name 'strandin' (Folch *et al.* 1951), given to similar material isolated from brain, is not used for the following reasons: (1) The report that strandin (Folch *et al.* 1951) does not contain appreciable neuraminic acid is now known to be in error (Chatagnon & Chatagnon, 1954; Daun, 1952; Rosenberg, Howe & Chargaff, 1956; Bogoch, 1956). (2) Other similarities in the composition of these substances have been demonstrated (Chatagnon & Chatagnon, 1954; Daun, 1952; Rosenberg *et al.* 1956; Bogoch, 1956). (3) The previously supposed difference in yield was found to be non-existent; when all the subfractions which were discarded by Klenk in his original procedure were taken into account, the yield of both substances was approx. 2 mg./g. of whole ox cerebral hemispheres (Bogoch, 1956).

RESULTS

Stepwise hydrolysis of brain ganglioside

Autohydrolysis. For recent suggestions on nomenclature for the sialic and neuraminic acids, see Blix, Gottschalk & Klenk (1957). The structure (I) proposed by Cornforth, Daines & Gottschalk (1957) for *N*-acetylneuraminic acid is probably very close to the true structure, although the molecular formula $C_{12}H_{21}O_{10}N$ for *N*-acetylneuraminic acid (Klenk & Faillard, 1954) is here used in all calculations.



(I)

It has been found that aqueous solutions of mucins and gangliosides undergo autohydrolysis, giving free neuraminic acid when heated at 100° (Blix, 1936; Folch *et al.* 1956; Bogoch, 1956). Whereas intact brain ganglioside has no reducing power, the neuraminic acid fraction liberated by autohydrolysis possesses a reducing power of 57% (expressed as glucose), and the non-diffusible residue of the ganglioside possesses no reducing power (Table 1). This suggests that the neuraminic acid is linked to the rest of the molecule through its reducing group. If samples of the autohydrolysate are taken at frequent intervals, and the reducing power is determined, the liberation of free reducing groups is seen to coincide quantitatively with the release of neuraminic acid (Bogoch, 1956). Thus it is this linkage which is cleaved during autohydrolysis.

The appearance of reducing groups is associated with the evolution of an absorption peak at 270 mμ during autohydrolysis. That this absorption may be related to a cyclization of neuraminic acid is suggested by the following experiment. An aqueous solution of crystalline neuraminic acid, obtained by treatment of diffusate I with cold methanol (1·1 mg./ml., birefringent needles, insoluble in cold methanol; N 4·5%, reducing sugar 61·4%), possesses no absorption peak at 270 mμ. If this solution is heated at 100° for 30 min. (as is done in the autohydrolysis of ganglioside), an absorption peak at this wavelength gradually appears (Fig. 1). The crystalline neuraminic acid is simultaneously converted into a pink amorphous material, now soluble in cold methanol, with unchanged reducing power. A similar change occurs spontaneously over a period of several weeks if the crystalline material is exposed to light and air at room temperature. This product, which has the same R_F as crystalline neuraminic acid, can be distinguished by the fact that it gives an immediate

Table 1. *Stepwise hydrolysis of brain ganglioside*

Procedure	Fraction	Dry wt. (as % ash-free ganglioside)	Composition of fraction (%)				
			Neuraminic acid	Nitrogen	Hexose	Reducing sugar	Galactosamine
	Ash-free ganglioside	100·0	30·3	2·9	24·0	0	10·0
Autohydrolysis at 100° for 18 min. followed by dialysis	→Diffusate I	20·8	88·0	4·42	0·74	57·0	2·5
	Residue *A*	81·0	13·0	2·7	—	—	—
Hydrolysis with 0·09 N-HCl at 100° for 50 min. followed by dialysis	→Diffusate II	17·0	33·0	4·24	21·3	57·3	14·2
	Residue *B*	64·0	3·0	1·99	31·3	—	9·6
Hydrolysis with 6 N-HCl at 100° for 5 min. followed by dialysis	→Diffusate III	23·4	—	2·36	72·6	39·3	16·6
	Residue *D*	31·8	0	—	—	—	—
Centrifuging	→Supernatant	0·14	—	—	—	—	—
	Precipitate	—	0	—	—	—	—
Solvent partition	→Upper phase (D_1)	4·3	—	2·9	17·0	2·0	0·13
	→Interphase	1·0	—	—	—	—	—
	→Lower phase	25·1	—	1·87	5·2	—	—

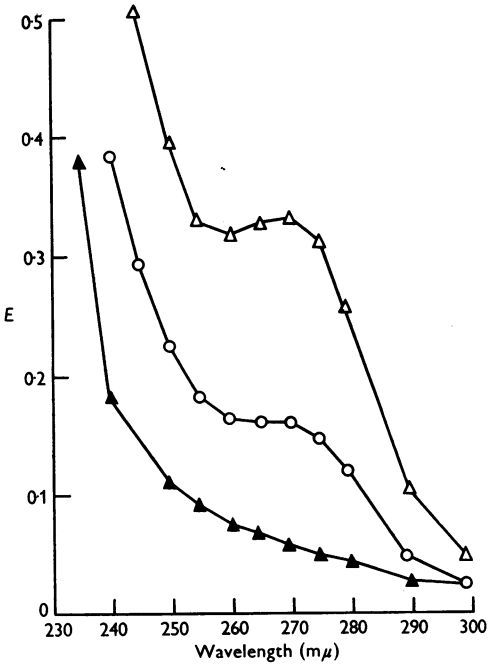


Fig. 1. The change in the ultraviolet spectrum of crystalline neuraminic acid on heating at 100°. ▲, No heating; ○, heated for 10 min.; △, heated for 30 min.

maximum red colour on spraying with Ehrlich's reagent and heating with steam, whereas crystalline neuraminic acid gives a maximum reaction only after heating for 10 min. This suggests that the change which occurs (possibly cyclization or polymerization) does not involve the reducing group.

Table 1 shows that diffusate I contains, in addition to neuraminic acid, 2·5 % of bound galactosamine (which is released in free form only after hydrolysis with N-HCl at 100° for 16 hr.) and hexose 0·74 %. This suggests that the neuraminic acid of brain ganglioside is bound to a neighbouring galactosamine molecule. Since the galactosamine of brain ganglioside is acetylated (Klenk & Lauenstein, 1953), and the amino group is thus not available for binding, it would appear likely that there is a glycosidic linkage between the reducing group of neuraminic acid and a hydroxyl group of galactosamine. Gottschalk (1956) has assumed that with some mucoproteins, neuraminic acid is linked to galactosamine by means of an *O*- or an *N*-glycosidic linkage. With brain ganglioside it would appear from the above that neuraminic acid is attached to galactosamine, and that it is attached by an *O*-glycosidic rather than an *N*-glycosidic linkage.

During the course of autohydrolysis the viscosity of the solution first decreases (to 42 % of its original value in 12 min.), increases to its original value in the next 15 min., then suddenly becomes so viscous that no flow occurs through an upright pipette with an internal diameter of 3 mm. The significance of this fact is taken up in the Discussion. The non-diffusible material remaining after autohydrolysis and dialysis is residue *A*. It accounted for 81 % of the original ganglioside (Table 1).

Hydrolysis of residue A. (1) Autohydrolysis of residue *A* (5 % aqueous solution, 100°, 30 min.) resulted in the conversion into dialysable form of only another 3 % of the original ganglioside. Thus about 30 % of the neuraminic acid of the intact molecule remains resistant to this treatment, and

requires hydrolysis with added acid to effect its removal. (2) Residue *A* was hydrolysed with added acid (0·09N-HCl, 100°, 50 min.) and then dialysed exhaustively. In this case the diffusate (diffusate II) contained a further 18·5% of the neuraminic acid, 24·2% of the galactosamine and 15% of the hexose of the intact ganglioside (Table 1). The hexose has been shown to be exclusively galactose by paper chromatography. The non-diffusible residue (residue *B*) still accounted for 64% of the original ganglioside. The analysis of residue *B* is also shown in Table 1. It may be noted that in both this and in the next hydrolytic step, there is an unavoidable destruction of neuraminic acid due to its acid lability.

Hydrolysis of residue B. Residue *B* was hydrolysed (6N-HCl, 100°, 5 min.) and then dialysed exhaustively, yielding diffusate III, which contained the balance of the recoverable neuraminic acid, galactosamine and galactose, plus some of the glucose of the original ganglioside (Table 1). It is noteworthy that this was the first point in the stepwise hydrolysis at which glucose was cloven from the ganglioside. The non-diffusible residue (residue *D*) contained the sphingosine and fatty acid of the original molecule, plus 8% of its hexose, but no appreciable galactosamine or neuraminic acid. Whereas the loss of galactosamine [in terms of the modified Elson & Morgan (1934) reaction] was 9·4% of the original up to the stage of residue *B*, a further 21·9% was lost in the brief hydrolysis of residue *B* with 6N-HCl.

Analysis of diffusates II and III. Samples of diffusates II and III were subjected to paper chromatography. In addition to distinct spots corresponding to neuraminic acid, hexosamine, glucose and galactose, two well-defined spots of slower mobility were present which corresponded to none of the above. That these slow-moving substances were oligosaccharides is suggested by the following experiments:

(1) Corresponding undeveloped areas of neighbouring duplicate strips of the same chromatogram were eluted with 5 ml. of water, and the eluates were examined by the reducing sugar and orcinol–sulphuric acid methods (Table 2). Control strips to detect substances eluted from the paper itself were taken from areas where there were no spots. The ratio 'orcinol sugar':'reducing sugar' indicated whether the sugars were free or bound. Furthermore, by determining the proportion of total hexose eluted from the area corresponding to glucose or galactose, an estimate could be obtained of the percentage of the total hexose of the fraction which was free. Diffusate II contained some 25% of its total hexose as free monosaccharide, the balance being bound in four different oligosaccharides, whereas diffusate III contained only 4% as free monosaccharide, the balance being bound in four different oligosaccharides. The recovery from the eluted spots in terms of the orcinol reaction of the original diffusate applied to the paper was 107% for diffusate II and 91% for diffusate III. From Table 2 it may be seen that spot 1 of diffusate III is an oligosaccharide. Other oligosaccharides are present in the first four spots of both diffusates, but are less apparent because of the presence of neuraminic acid and galactosamine. Thus spots 1 and 2 of diffusate II, and 2 and 3 of diffusate III, have low values for orcinol:reducing sugar because they contain neuraminic acid, which has a high reducing value but gives no hexose

Table 2. *Chromatographic demonstration of oligosaccharides and monosaccharides in diffusates II and III*

Where two spots are listed opposite neuraminic acid, these spots travelled close together opposite the single neuraminic acid spot.

Spot (in order of increasing mobility)	Orcinol sugar as galactose (μg.) (*a*)	Reducing sugar as galactose (μg.) (*b*)	Ratio *a*/*b*
Diffusate II			
(1) Neuraminic acid	280	301	0·93
(2) Neuraminic acid	40	100	0·40
(3) Tail of 2	27	83	0·33
(4) Hexosamine	88	198	0·45
(5) Tai of 4	10	30	—
(6) Galactose	150	119	1·26
Total hexose (μg.	595		
Diffusate III			
(1) Unknown	146υ	440	3·32
(2) Neuraminic acid	92	80	1·15
(3) Neuraminic acid	58	75	0·77
(4) Hexosamine	84	104	0·81
(5) Galactose	15	30	—
(6) Glucose	54	30	—
Total hexose (μg.)	1763		

reaction with orcinol. Similarly, spot 4 of both diffusates has a low orcinol:reducing sugar value because of the failure of hexosamine to give an orcinol reaction. The hexose present in all of these spots is bound, for if free, it would be opposite spots 5 or 6.

(2) The presence of oligossacharides is supported by the further hydrolysis of the diffusates and chromatography of the products. Hydrolysis of diffusate II with N-HCl resulted in the disappearance of the oligosaccharide spots, and the appearance of galactose as the sole hexose. With diffusate III, both galactose and glucose were present, but they required hydrolysis with stronger acid (2N-HCl) in order to liberate the monosaccharides. Evidence for this statement is as follows: after diffusate III was hydrolysed with N-HCl (sealed tube, 100°, 16 hr.), the product could be partitioned by ion-exchange chromatography into neutral and amino-sugar fractions. The neutral sugar fraction still showed oligosaccharide spots on paper chromatography. However, when this neutral sugar fraction was treated for varying periods with 2N-HCl (sealed tube, 100°) the oligosaccharides were hydrolysed. The resultant evolution of reducing groups is shown in Fig. 2.

Amino sugar fraction of diffusates II and III. Hydrolysis of diffusate III (N-HCl, sealed tube, 100°, 16 hr.), followed by Dowex-50 chromatography, yielded an amino sugar fraction consisting of large birefringent crystals arranged spherically. These crystals represented 20·5% of diffusate III and contained 5·6% of N and 78% of hexosamine. On chromatography there was only one spot, corresponding to galactosamine.

Hydrolysis of this amino sugar fraction with 6N-HCl (100°, sealed tube, 10 min.), followed by paper chromatography, revealed that the galactosamine spot, though still present, was diminished in intensity and a new spot with 50% faster mobility, which also reacted with ninhydrin, was now present.

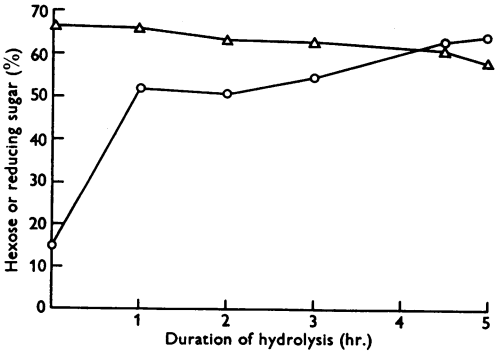


Fig. 2. Hydrolysis of neutral sugar fraction of diffusate III with 2N-HCl at 100° for varying periods: △, % hexose (as galactose); ○, % reducing sugar.

The same treatment of a pure sample of galactosamine yielded identical chromatographic results. The appearance of this second spot on treatment with 6N-HCl would appear to be related to the simultaneous loss of the ability to give the modified Elson & Morgan (1934) reaction for hexosamine which had previously been observed in the hydrolysis of residue *B* with 6N-HCl. The derivative which is formed may be a cyclized or isomerized product of galactosamine.

The hexosamine of this preparation of brain ganglioside was identified as galactosamine (see Methods), and the content of galactosamine found to be 10% by weight of the intact ganglioside.

Analysis of residue D. Residue *D* comprised the non-diffusible material remaining after hydrolysis of residue *B* with 6N-HCl (Table 1). It was a hazy, opalescent solution containing insoluble particles. The insoluble material was separated by centrifuging, then partitioned between chloroform–methanol (2:1, v/v) and water. The analyses of the resultant phases are shown in Table 1.

The upper phase (D_1) contained 2·9% N, of which 2·3% was free amino N. The hexose content was 17%. There was essentially no hexosamine. and the reducing sugar was only 2%. This suggested that D_1 contained sphingosine with some hexose attached. Evidence that this was the case follows from the results of further hydrolysis (N-HCl, sealed tube, 100°, 16 hr.). (1) The hydrolysate was centrifuged; the resultant supernatant contained 69% of the original hexose, and the precipitate contained 100% of the original N but only 10% of the original hexose (precipitate N 4·4%). (2) The reducing sugar of D_1 (as equivalents of galactose) rose from 2% before, to 19·2% after, hydrolysis. (3) Whereas chromatography of unhydrolysed D_1 revealed only one spot with very rapid mobility, and no free hexose, hydrolysed D_1 showed only glucose (no galactose).

An unexpected finding was the presence in the precipitate of hydrolysed D_1 of a reducing sugar value of 11·1% (as galactose), which was not accounted for by the hexose (orcinol) content of only 1·7%. This finding, together with the presence of 2% of reducing sugar in unhydrolysed D_1 (from which all free hexose or hexose fragments had been removed by dialysis), suggests the possibility that the sphingosine of brain ganglioside (Klenk, 1942; Rosenberg & Chargaff, 1956) is modified to the extent that it contains a reducing group. A possible function of such a reducing group is discussed in the formulation of the structure of the repeating unit.

Quantitative nature of stepwise hydrolysis

The stepwise procedure outlined was found to be reproducible. Recovery in terms of hexose and N (adding all of the subfractions) was 96·6 and

96·0 % respectively. Furthermore, the constituents were repeatedly released in the following order: neuraminic acid, galactosamine, galactose, glucose, sphingosine and fatty acids. If the hydrolytic procedure was varied, the order of release was maintained. Thus if instead of subjecting residue *B* to hydrolysis with 6 N-HCl, it was hydrolysed with 0·09 N-HCl (100°, sealed tube, 3 hr.), then dialysed exhaustively, another 11·7 % of the original ganglioside was released in diffusible form. This diffusate was found to contain 38 % of hexose (galactose only by paper chromatography) and 24·8 % of galactosamine, representing essentially the balance of the galactosamine of the intact molecule. The non-diffusible residue (B_1) still contained 27·8 % of hexose, in addition to all of the sphingosine and fatty acid. It is important to note that residue B_1 was still soluble in water, indicating that neither fatty acid nor ceramide nor cerebroside was freed in appreciable amounts, since all of these are insoluble in water.

Cleavage of a cerebroside from brain ganglioside

The hydrolyses of residues *A* and *B* with 0·09 N-HCl, which removed galactose but not glucose, suggested that a glucocerebroside might be isolated. After several attempts, optimum conditions were defined as follows: residue *A* was hydrolysed with 0·09 N-HCl (sealed tube, 100°, 16 hr.), then dialysed. The resulting non-diffusible residue (residue *C*), still water-soluble, was further hydrolysed (0·09 N-HCl, sealed tube, 100°, 5 hr.). The hydrolysate, which now contained a tan, insoluble material, was dialysed. This water-insoluble material was separated by centrifuging, dried *in vacuo* and dissolved in warm methanol–chloroform (9:1, v/v) to give a clear solution (12 % remains as insoluble black residue which is discarded). When this solution (40 mg./ml.) is allowed to cool to room temperature, birefringent spherocrystals are formed, amounting to 26–30 % by weight of the intact brain ganglioside. These crystals contain no neuraminic acid or galactosamine. The analysis, compared with that expected for a glucostearocerebroside monohydrate (Rosenheim, 1914), is as follows: [Found: C, 67·13; H, 10·95; N, 1·86; hexose (as glucose), 21·8. Calc. for $C_{42}H_{81}O_8N, H_2O$: C, 67·65; H, 11·14; N, 1·88; hexose (as glucose), 22·0 %].

The material is insoluble in water, slightly soluble in pure chloroform, very soluble in chloroform–methanol (2:1, or 3:1, v/v). It shows slight liquefaction at 165°, then melts sharply at 172–174°. The iodine number is 23·2. In chloroform–methanol (2:1, v/v) (1 mg./ml.) it is laevorotatory ($[\alpha]_D^{20} - 2·08$). The infrared spectrum is shown in Fig. 3. A sample of the crystalline material gave the following spacings by X-ray diffraction: 2·41, 4·10, 9·1, 10·4, 15·6, 21·1, 30·6, 49·2, 55·5 and 63·5 Å.

Hydrolysis in N-HCl (sealed tube, 100°, 16 hr.) liberated the hexose quantitatively. This was shown to be exclusively glucose by paper chromatography. The remaining water-insoluble residue contained 2·3 % of N, as expected for sphingosine and fatty acid in equimolar ratio. It is yet to be established whether the base is sphingosine or dihydrosphingosine (Rosenberg & Chargaff, 1956; Carter & Norris, 1942).

The melting point, specific rotation, iodine number and elementary analysis distinguish this substance from the previously described cerebrosides phrenosine, kerasine and nervone (Deuel, 1951). A glucostearocerebroside has not previously been described. Its presence as the basic constituent of brain ganglioside would be consistent with the demonstration by Klenk (1942) that the constituent fatty acid of brain ganglioside is stearic acid. The name gangliocerebroside is proposed for this new substance.

Cleavage of a hexodicerebroside from brain ganglioside

With the demonstration that brain ganglioside could be degraded to a glucocerebroside, it became of interest to establish the nature of the linkage of

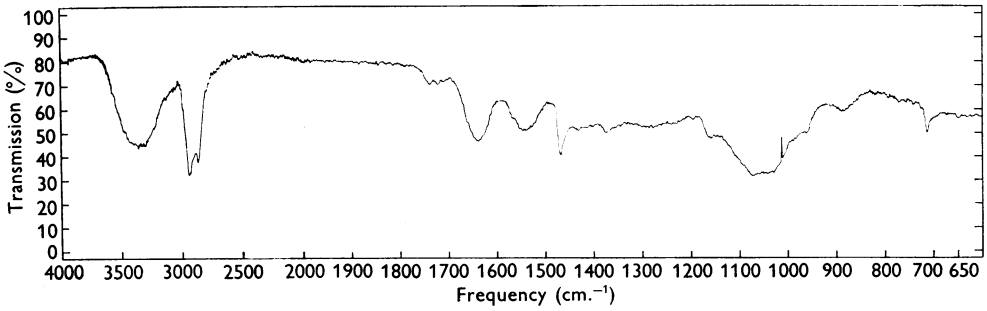


Fig. 3. Infrared spectrogram of gangliocerebroside (Perkin–Elmer double-beam automatic-recording spectrophotometer, NaCl prism).

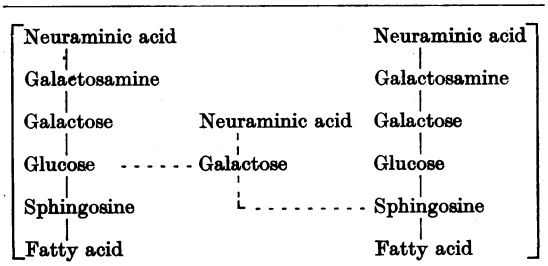
the individual cerebroside molecules to each other. Since in the preparation of the glucocerebroside no insoluble material appeared until the hydrolysis of residue *C*, it seemed likely that a fraction would be partitioned from residue *C* which was simply a dicerebroside or a dicerebroside with a hexose, or a galactosamine or possibly a neuraminic acid molecule as the bridge between the two individual cerebrosides. The analysis of such a fraction might indicate clearly which of the above four possibilities was correct. It was, of course, possible that the analyses for the above constituents would indicate a random arrangement.

When residue *C* was dried *in vacuo* and repeatedly washed with warm methanol–chloroform (9:1, v/v) a soluble fraction was obtained which crystallized in a manner similar to the gangliocerebroside. The yield of this new material represented 90% by weight of residue *C* and 41% by weight of the intact brain ganglioside. Galactosamine was absent, and the neuraminic acid content was only 1·05%. The hexose content and elementary analysis strongly suggested that this substance was in fact a single or polymerized hexodicerebroside [Found: C, 66·02; H, 10·54; N, 1·77; hexose (as glucose), 30·2. Calc. for $C_{90}H_{170}O_{20}N_2,H_2O$: C, 66·01; H, 10·51; N, 1·71; hexose (as glucose), 29·8%].

This substance differed also in melting point from the glucocerebroside; it liquefied slightly at 175° and melted sharply at 182–184°.

DISCUSSION

The determination of structure by the quantitative stepwise procedure outlined suggests that what is being studied is an organized arrangement of constituents bound together in firm covalent linkage. This is indicated by the stoicheiometric appearance of reducing groups as constituents are successively cloven from glycosidic binding, and by the constant order of release of diffusible constituents. The constant order of release of constituents observed in repeated stepwise hydrolyses, i.e. neuraminic acid, galactosamine, galactose, glucose, sphingosine and fatty acids, is suggestive of a structural sequence. Furthermore, the data from the analysis of the fractions shown in parentheses present evidence for the existance of the following groupings: neuraminic acid–galactosamine (diffusate I); galactosamine–galactose–glucose (diffusates II and III; residue B_1); glucose–sphingosine (D_1); glucose–sphingosine–fatty acid; sphingosine–fatty acid and hexodicerebroside. These data, considered with the percentage composition of the constituents of the intact ganglioside, suggest the repeating unit (II) [Found: N, 2·9; neuraminic acid, 30·3; hexose, 24; galactosamine, 10. Calc. for the structure (II): N, 2·96; neuraminic acid, 30·5; hexose, 24·4; galactosamine, 10·8%].



Mol.wt. of repeating unit: 3314.

(II)

In (II) the linkages which have not yet been positively identified are shown by interrupted lines. Since there would be a shortage of reducing groups required to unite all of the members of the repeating unit in the manner suggested by the hydrolytic studies, the evidence suggesting a sphingosine molecule modified in that it possesses a reducing group is pertinent. The empirical observation that about 60% of the total neuraminic acid can be removed by autohydrolysis, but that to free the remainder requires more vigorous acidic hydrolysis, is in agreement with the more peripheral localization of 2 of the 3 moles of neuraminic acid/unit.

A repeating unit of this molecular size would appear to be unique, and presents interesting problems with regard to the spatial organization of the macromolecular polymer. By simply arranging the repeating units linearly, a fibrous structure would be realized. The physical properties suggest, however, a globular molecule. Thus in aqueous solution flow birefringence is not marked and, on autohydrolysis, the initial fall in viscosity followed by a later increase suggests the uncoiling of a globular unit, followed by the aggregation of linear fragments. A further empirical point requiring some attention is the fact that whereas brain ganglioside is remarkably soluble in water (despite its high lipid content) it is also soluble in organic solvents. The sequence indicated by this study would permit the coiling of the polymerized units into, for example, a helix, in such a way that lipid-soluble constituents could be enfolded by water-soluble constituents in aqueous solution. In organic solvents, the coiling might occur in the opposite direction, such that the lipid-soluble constituents 'faced' the solvent. It may also be noted that the extended linear form of the brain ganglioside could function admirably in transport phenomena at the nerve-cell surface, where it would be useful to present water-soluble constituents in one direction and lipid-soluble constituents in the other. The relation of neuraminic acid to the

surface receptor substance of cells for the attachment of viruses (Klenk & Lauenstein, 1952; Gottschalk, 1954, 1955; Klenk, Faillard & Lempfrid, 1955) and the demonstration that this preparation of brain ganglioside is an inhibitor of the haemagglutination reaction of influenza virus (Bogoch, 1956, 1957) are of interest in relation to the present suggestions for the overall structure.

SUMMARY

1. A preparation of brain ganglioside, homogeneous by electrophoretic and ultracentrifugal studies, has been examined by a quantitative stepwise hydrolytic procedure.

2. Diffusible products have been quantitatively separated from those which are non-diffusible at each hydrolytic step by exhaustive dialysis.

3. The diffusible and non-diffusible fractions thus obtained have been analysed in terms of dry weight, nitrogen, neuraminic acid, hexosamine, hexose and reducing sugar, partitioned by paper-chromatographic and ion-exchange procedures, and the subfractions further hydrolysed and analysed.

4. The mode of linkage of the neuraminic acid component has been demonstrated.

5. Oligosaccharide fragments were isolated, the hexoses shown to be exclusively galactose and glucose, and the hexosamine, galactosamine.

6. The galactosamine content has been found to be 10%, and a reaction of galactosamine on heating with concentrated acid has been observed.

7. A glucocerebroside has been cloven from the intact ganglioside, characterized and named gangliocerebroside.

8. A hexodicerebroside has also been isolated.

9. The constant order of release of constituents, together with the evidence regarding the structure of isolated fragments, has been used to formulate the structure of the repeating unit of the molecule.

10. Observations have been made relevant to the overall structure and orientation of the macromolecular glycolipid.

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