coenzyme Q is coenzyme Q_{10}, the greater part being coenzyme Q_9. Whether coenzyme Q_9 would behave differently in the rat body will remain uncertain until [^{14}C]coenzyme Q_9 becomes available. However, two arguments can be advanced in favour of the validity of our experiments. The homologues coenzyme Q_{10} and coenzyme Q_9 are both synthesized in rat liver (Wiss, Gloor & Weber, 1961), and, in addition, rat brain contains a relatively high concentration of coenzyme Q_{10} (Green, 1961). If transport from liver were to be the source of coenzyme Q in other tissues, then coenzyme Q_{10} should also have been incorporated into brain.

SUMMARY

1. Administration of [^{14}C]coenzyme Q_{10} by oral or intracardial routes to rats resulted in its exclusive incorporation into liver. Although this radioactivity rapidly decreased, it was not transferred to other tissues even after 48 hr.

2. The greater part of the radioactivity in the unsaponifiable lipids of liver was in the form of unchanged coenzyme Q_{10}, even after 24 hr. Under these conditions, administered [^{14}C]coenzyme Q_{10} was not converted into its cyclic isomer, ubichromenol.

3. The coenzyme Q content of rat foetuses increased during development. Blood coenzyme Q can pass through placenta and become incorporated into the foetuses.

4. Injected [^{14}C]coenzyme Q_{10} is rapidly removed from rat blood. Dietary coenzyme Q seems to be the source of blood coenzyme Q and therefore assumes indirect importance in embryonic development.

5. Several rat tissues are capable of synthesizing [^{14}C]coenzyme Q from [2-^{14}C]mevalonic acid.

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Physicochemical Properties of Ox-Brain Gangliosides

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Gangliosides are the group of sialomucolipids that are derived from cerebral tissues. Methods of preparation and purification of gangliosides and recent advances in the elucidation of their chemical structure have been reviewed by McIlwain (1961*a*) and Rapport & Norton (1962). Fractionation of gangliosides has demonstrated the existence of several molecular species differing in their chemical composition. The differences between species, however, are relatively small and simple molar proportions of the chemical constituents have been assigned to each. Thus each has been shown to have a polar

moiety consisting of a polysaccharide chain with 1 or more *N*-acetylneuraminic acid residues/molecule together with a non-polar ceramide moiety/molecule, predominantly sphingosine–stearic acid in ox-brain gangliosides (Trams, Giuffrida & Karmen, 1962). Gangliosides isolated from brains of other animals show some differences in their chemical composition.

The neutral equivalents measured for gangliosides from human and ox brain fall in the range 1000–1500, values that agree well with the calculated molecular weights (Klenk & Gielen, 1960; Kuhn *et al.* 1960). Further, sedimentation of gangliosides in *NN*-dimethylformamide has given similar values for the minimum molecular weight. Molecular weights of 180000–250000 or more have been measured for gangliosides in aqueous solution (Folch, Arsove & Meath, 1951; Rosenberg & Chargaff, 1958; Trams & Lauter, 1962), from which it has been concluded that gangliosides aggregate in aqueous media to form micelles as a result of the presence of both hydrophobic and hydrophilic groups in the molecules (Klenk & Gielen, 1960). This has been confirmed by the decrease in size of the ganglioside units observed in non-aqueous or only partly aqueous solutions, which is attributed to dissociation of micelles into individual molecules or small groups of molecules (Klenk & Gielen, 1960; Trams & Lauter, 1962).

The present paper is concerned with the physical properties of ox-brain gangliosides in aqueous media and the importance of these properties in relation to the previously described activity of gangliosides in biological systems (McIlwain, 1961*b*; McIlwain, Woodman & Cummins, 1961; van Heyningen & Miller, 1961; Doery & North, 1961).

EXPERIMENTAL

Preparation of gangliosides

The method employed was that described by Booth (1962) for large-scale preparations which is based on the procedure of Long & Staples (1959), which followed that of Folch, Lees & Sloane-Stanley (1957*a*).

Acetone-drying before extraction of cerebral tissues has been found to minimize the amount of peptide material in the final preparation (Svennerholm, 1956). Fresh ox-brain grey matter was therefore first blended in a top-drive macerator for 2 min. with 3 ml. of acetone/g. and then with 1 ml./g., and the suspension filtered through a Buchner funnel with Whatman no. 43 papers. Pressure was applied to the residue on the funnel to remove as much of the acetone extract as possible. The residue was air-dried overnight and then finely ground in a pestle and mortar. The yield of acetone-dried powder in a typical preparation was 173 g./kg. of original grey matter. A weight of acetone-dried powder equivalent to 500 g. of fresh tissue was agitated for 60 min. with 10 l. of chloroform–methanol (1 : 1, v/v) in a Vibromix stirrer (Shandon Scientific Co. Ltd., London). The extract was filtered through a fluted Whatman no. 1 paper, and to the filtrate was added 5 l. of chloroform and 3 l. of 0·1 M-KCl. The mixture was agitated for a further hour, the emulsion being maintained throughout this time. After standing for several hours, the clear upper phase which settled out was siphoned off and dialysed against running tap water at about 10° for 1–2 days. During dialysis the contents of each sac separated into a clear solution above an opaque chloroform emulsion. On completion of dialysis, the clear solution was withdrawn from each sac, leaving behind the chloroform emulsion, which was treated separately. The volume of the clear solution was reduced to about 1 l. in a rotary evaporator operated at 50–60°. The pH of the concentrated solution was finally adjusted to 2·6 with HCl. The solution was exhaustively dialysed against distilled water and freeze-dried. The yield of white fluffy powder obtained from 500 g. of grey matter varied between 600 and 750 mg. The chloroform emulsion, which was similarly treated, did not require adjustment of its pH. It accounted for some 20% of the total dry weight of the upper phase.

Further treatment of the ganglioside preparation

The removal of associated small ions was achieved by passage of water solutions through a column of Amberlite MB-3 mixed-bed resin (Rohm and Haas Co., Philadelphia, Pa., U.S.A.). The de-ionized gangliosides were then freeze-dried.

Ultrasonic irradiation was performed with the MSE ultrasonic disintegrator unit (Measuring and Scientific Equipment Ltd., London) by the method described by Saunders, Perrin & Gammack (1962).

Analytical methods for gangliosides

N-*Acetylneuraminic acid*. This was determined with Bial's orcinol–$FeCl_3$–HCl reagent, by the method of Balakrishnan & McIlwain (1961), with a commercial preparation of *N*-acetylneuraminic acid (L. Light and Co. Ltd., Colnbrook, Bucks.) as a standard.

Phospholipid phosphorus. This was determined as orthophosphate as described by Long (1943) after digestion by the method of King (1932).

Paper chromatography of gangliosides. This was done by the ascending method of van Heyningen & Miller (1961) on Schleicher und Schüll (Dassel, Germany) 2045 B paper with the solvent mixture di-isobutyl ketone–acetic acid–water (40 : 30 : 7, by vol.). The solvent system tetrahydrofuran–di-isobutyl ketone–10% (v/v) acetic acid (50 : 5 : 9, by vol.) (W. E. van Heyningen, personal communication) was also used. The developed chromatograms were stained with Cresyl Fast Violet, excess of dye being removed by washing in 10% (v/v) acetic acid.

Thin-layer chromatography. This employed the apparatus of Stahl (Camlab, Cambridge) and Kieselgel G (Merck). Chromatograms were developed in the solvent system chloroform–methanol–water (60 : 35 : 8, by vol.) as described by Wagner, Hörhammer & Wolff (1961) for chromatography of sphingolipids, but with the inclusion of dilute aq. (1%, v/v) ammonia (sp.gr. 0·88), which improves the separation of certain ganglioside components (Müldner, Wherrett & Cumings, 1962). Bial's orcinol reagent, as prepared for the assay of *N*-acetylneuraminic acid, was used

as spray reagent for gangliosides. The plates were heated at 120° for 10–15 min. after spraying. Identification of other lipids in the ganglioside preparations was carried out by chromatography in the solvent system chloroform–methanol–water (60 : 25 : 4, by vol.), with spray reagents specific for different groups on the lipids according to the method of Wagner *et al.* (1961).

Fractions were isolated from thin-layer chromatograms by the method described by Wherrett & Cumings (1963), and the molar ratio of *N*-acetylneuraminic acid to sphingosine was determined for each fraction by Dr J. R. Wherrett.

Physicochemical methods

Sedimentation analysis. This was performed in a Spinco model E instrument fitted with a schlieren phase plate. Experiments were done at temperatures in the range 18–22°, the temperature for each experiment being controlled to ±0·1° by the R.T.I.C. controller (Spinco Inc., Palo Alto, Calif., U.S.A.). Sedimentation coefficients were computed from plots of ln (x) against t at constant speed and corrected to water at 20° by the usual expressions to give $S_{20,w}$ values. Sedimentation of gangliosides in *NN*-dimethylformamide was performed in the synthetic-boundary cell (Schachman & Harrington, 1954).

Diffusion measurements. These were made in the diffusion apparatus described by Saunders (1953) but subsequently modified, with a newly designed cell (R. Fleming, unpublished work). Diffusion coefficients were determined at 25±0·1° by the Gouy interference method (Gosting, 1956). Differential diffusion of a 1·2% (w/w) solution of gangliosides in water into a 0·2% (w/w) solution yielded steady values after diffusion had proceeded for 24 hr. Observed values were corrected to water at 20° and the mean $D_{20,w}$ was obtained from eight interference patterns, which gave values agreeing to within 1%.

Moving-boundary electrophoresis. This was performed in a Hilger and Watts apparatus with schlieren optics. Measurements of electrophoretic mobility were made at 1·5°.

Electrical-conductivity measurements. These were made on a Pye conductance bridge (W. G. Pye and Co. Ltd., Cambridge) with a Shedlovsky conductivity cell (Longsworth, Shedlovsky & MacInnes, 1939). In studying the effect of dilution on conductivity, measurements were made on gangliosides that had previously been de-ionized by passage through a column of Amberlite MB-3. The large size of the micelles prevented the removal of appreciable amounts of ganglioside by the resins.

Viscosity measurements. These were made in a Cannon–Fenske no. 25 viscometer (B.S. 188: 1957) at 25±0·01°. A mean $\eta_{sp.}/c$ was obtained from four determinations on concentrations of gangliosides in water of between 1·1 and 1·2% (w/v).

Surface-tension measurements. These were made by the Wilhelmy method, with a platinum strip suspended from a direct-reading electrically-operated balance (Padday, 1957). The surface tension of ganglioside solutions in water changed with time. Equilibrium values were read at each concentration after 4 hr.

Gel-filtration studies. These were carried out with columns of Sephadex G-25 (Pharmacia, Uppsala, Sweden), prepared with water or 0·1M-tris–HCl buffer, pH 8·0, in 0·2M-NaCl according to the manufacturers' recommendations.

RESULTS

Chemical characteristics of the ganglioside preparation

The preparations of gangliosides on which previous physicochemical determinations have been made were from whole brain. As most of the ganglioside is located in the grey matter, a preparation made from grey matter derived from ox-brain has been examined in the present investigation. The method of large-scale preparation of ox-brain gangliosides described by Booth (1962) has been shown to give good yields. Three batches were made by this method with the modification that the chloroform emulsion formed during the preliminary dialysis was not included in the final material collected. The batches contained 27·2–28·2% (w/w) of *N*-acetylneuraminic acid and had phosphorus contents of less than 0·1% (w/w). Grey matter used for the preparation was pre-extracted with acetone, a procedure which has been shown to minimize the peptide content of the gangliosides.

Paper chromatography of the preparation in the di-isobutyl ketone–acetic acid–water solvent system showed resolution into two components: a slow-migrating spot which was metachromatic and a fast-migrating spot which did not show metachromasy on staining with Cresyl Fast Violet (cf. van Heyningen & Miller, 1961). Separation into at least five components was obtained with the tetrahydrofuran–di-isobutyl ketone–water solvent system: three slow metachromatic spots and two fast non-metachromatic spots. The spots were, however, poorly resolved and had low R_F values, making estimation of their relative amounts difficult.

On thin-layer chromatography, the ganglioside preparation showed five well-resolved components, all staining purple with the orcinol reagent, three minor components with low R_F values, a major component with a greater R_F, and a faster-migrating component present in intermediate amount (Fig. 1*a*). Hydrolysis of the gangliosides in 0·05N-hydrochloric acid for 30 min. at 80° caused a marked increase in the amount of the fastest-migrating component with a concomitant decrease in the relative amount of the component with intermediate R_F (Fig. 1*b*). The three slow-moving components appeared to be unchanged in amount. The fastest-moving component therefore corresponds to the ganglioside with 1 *N*-acetylneuraminic acid group/ceramide group, which was shown by Svennerholm & Raal (1961) to be the predominant ganglioside after mild acid hydrolysis. Three fractions were isolated from the thin-layer chromatograms: the fastest-moving component (fraction III), the component with intermediate R_F (fraction II) and the three slow-moving components together (fraction I). The molar ratios of *N*-acetyl-

neuraminic acid to sphingosine for the three fractions (see Table 2) confirm that fraction III consists of gangliosides with 1 *N*-acetylneuraminic acid group/ceramide group, whereas fractions I and II appear to be mixtures of ganglioside species with a different composition. The magnitude of the ratios for fractions I and II may find explanation in the observation of Kuhn, Wiegandt & Egge (1961) that certain fractions of gangliosides isolated from ox-brain contain as many as 2 and 3 *N*-acetylneuraminic acid residues/ceramide group.

Thin-layer chromatography in solvent sytems designed for the separation of phosphatides (Wagner *et al.* 1961) has led to the identification of the lipid impurities in the ganglioside preparation. Chromatographic separations were unaffected by the gross excess of gangliosides needed to be applied to the chromatograms, gangliosides moving only a short distance from the origin. Spots corresponding to lecithin, lysolecithin and cerebrosides were identified, but no ninhydrin-positive lipids could be detected. These contaminants were estimated to constitute less than 1% of the total weight of lipid. The chloroform emulsion excluded from the preparation contained a large ninhydrin-positive component, which was orcinol-negative, migrating just ahead of the gangliosides. This could not be identified with any of the known cerebral lipids. The chloroform emulsion had less than 10% (w/w) of *N*-acetylneuraminic acid and a higher phosphorus content and nitrogen content than the main upper-phase lipid material, so that its exclusion lessened the impurities in the final ganglioside preparation.

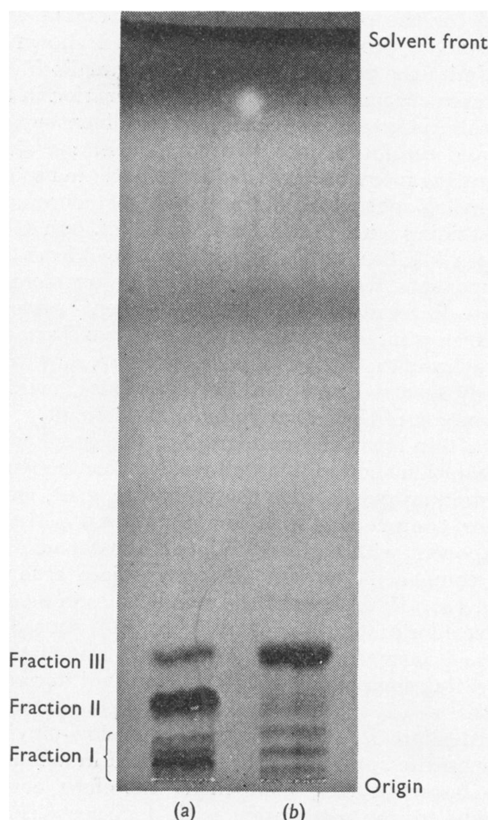

Fig. 1. Thin-layer chromatography of the ganglioside preparation in the solvent system chloroform–methanol–water–aq. ammonia (sp.gr. 0·88) (70 : 30 : 4 : 1, by vol.). (*a*) Untreated and (*b*) after hydrolysis with 0·05N-HCl at 80° for 30 min. Gangliosides were detected with Bial's orcinol reagent. Details are given in the text. The fraction numbers apply to ganglioside fractions eluted from chromatograms of the untreated material (see Table 2).

The material used in the present study contained only trace amounts of contaminating lipids, but it consisted of at least five distinct grey-matter gangliosides, being a group of sialomucolipids of closely related chemical composition.

Physical properties of the gangliosides

Sedimentation of a 1% (w/v) solution of the ganglioside preparation in *NN*-dimethylformamide gave a sedimentation coefficient corrected to water at 20° ($S_{20,w}$) of 0·53s (Table 1). Thus the minimum molecular weight for ox-brain gangliosides in *NN*-dimethylformamide falls in the range estimated for human brain gangliosides, namely 1000–2000 (Klenk & Gielen, 1960). Determination of the neutral equivalent by titration gave a value of 1520 (Table 1).

When the ganglioside preparation was sedimented in water, considerably higher sedimentation coefficients were measured. Sedimentation of 1% (w/v) solutions of samples of each of the three batches which made up the whole preparation yielded a mean value for $S_{20,w}$ of 10·3s (S.D. 0·2s; S.E.M. 0·5s). A marked concentration-dependence for sedimentation coefficient was observed, the $S_{20,w}$ values increasing with decreasing concentration (Fig. 2). Extrapolation to zero concentration gave a value for $S^0_{20,w}$ of 11·8s. The sedimentation patterns showed slight asymmetry, a shoulder being apparent on the leading edge of the single schlieren peak after prolonged sedimentation (Fig. 2). The shoulder decreased in apparent area with increasing concentration, probably as the result of the Johnston & Ogston (1946) effect observed with other macromolecular systems. The shoulder was enlarged in one preparation examined which contained 0·92% of phosphorus, yielding a characteristic pauci-polymolecular distribution; the $S_{20,w}$ of this second component was 13·6s.

The three fractions isolated from thin-layer chromatograms were separately dissolved in water and examined in the ultracentrifuge. Fraction I

Table 1. *Physical data for the ganglioside preparation*

Details are given in the text.

(*a*) Neutral equivalent		
1% (w/v) solution titrated against 0·10N-NaOH		1520
(*b*) Sedimentation coefficients	$S_{20,w}$ (s)	
1% (w/v) solution in *NN*-dimethylformamide		0·53
1% (w/v) solution in water		10·3±0·2 (3)
(*c*) Diffusion coefficient		
1·2% (w/w) solution in water into 0·2% (w/w) solution in water	$10^7 D_{20,w}$ (cm.²/sec.)	3·78
(*d*) Intrinsic viscosity	$\eta_{sp.}/c$ (g./100 ml.)$^{-1}$	0·0273±0·009 (4)
1·1–1·2% (w/v) solutions in water		
(*e*) Partial specific volume	$\bar{v}$ (ml./g.)	0·78
1% (w/v) solution in water		
(*f*) Micellar weights in water		
(i) calculated from (*b*), (*c*) and (*e*)		309 000
(ii) calculated from (*b*), (*d*) and (*e*)		257 000
(iii) calculated from (*c*) and (*d*)		446 000
(*g*) Electrophoretic mobility		
1% (w/v) solution in barbiturate buffer, pH 8·6 and *I* 0·1	$10^5 u_{desc.}$ (cm.²/sec./v)	8·1

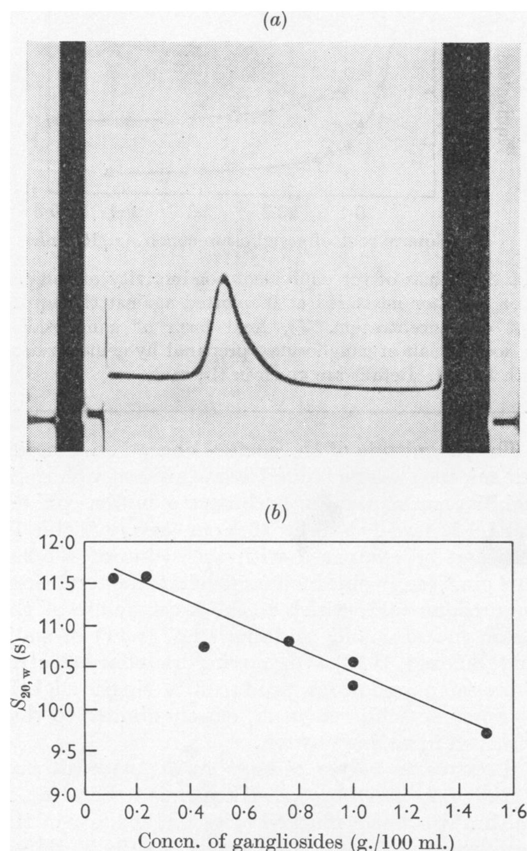

Fig. 2. (*a*) Sedimentation pattern for 1% (w/v) solution of the ganglioside preparation in water. The rotational speed was 42040 rev./min. The temperature was 21·5±0·1°. (*b*) Values of $S_{20,w}$ for the ganglioside preparation plotted against concentration. Details are given in the text.

gave a higher value for $S_{20,w}$ and fractions II and III lower values than those obtained for the unfractionated material at corresponding concentrations (Table 2).

The intrinsic viscosity, $\eta_{sp.}/c$, measured for the gangliosides was 0·0273 (g./100 ml.)$^{-1}$ at 25° (Table 1), giving an Einstein (1906) number of 3·5. This indicates that the micelles are spheroidal in shape. The micellar weight calculated from sedimentation and diffusion data by the Svedberg equation (Svedberg & Pedersen, 1940) gave a value of 309000. The micellar weight obtained from viscosity and diffusion data was 446000, and from viscosity and sedimentation data was 257000. These calculations were based on the method of Scheraga & Mandelkern (1953), by using a value of β equal to $2{\cdot}16 \times 10^6$ which is applicable to rigid impermeable spheres with axial ratios between 1 and 5 (Schachman, 1959).

The gangliosides could still be sedimented at a concentration of 0·02%, which was the limit of detection by the schlieren optical system in the ultracentrifuge cell with a light-path of 12 mm. However, measurement of the surface tension of solutions in water over a range of concentrations down to 0·002% revealed a marked change in the slope of the plot of surface tension against concentration occurring at between 0·015 and 0·02%, apparent as a minimum in the curve (Fig. 3). This represents the region containing the critical micelle concentration where the micelles are largely dissociated into smaller units, probably monomers. A characteristic minimum occurring near this concentration has also been observed with soap micelles and is due to impurity solubilized by the micelles (Miles & Shedlovsky, 1944). Gangliosides exhibited little surface activity. Concentrated solutions in

Table 2. *Sedimentation coefficients of ganglioside fractions*

Fractions isolated from thin-layer chromatograms of the ganglioside preparation (see Fig. 1) were pooled and the molar ratios of *N*-acetylneuraminic acid to sphingosine determined (see Wherrett & Cumings, 1963). Sedimentation coefficients were determined for the fractions dissolved in water and compared with values obtained for the unfractionated material at the same concentration. The latter values (in parentheses) were obtained from the plot of sedimentation coefficient against ganglioside concentration (Fig. 2*b*).

Fraction no.	Molar ratio of *N*-acetylneuraminic acid to sphingosine	Concn. in water (g./100 ml.)	$S_{20,w}$ (s)
I	2·4	0·15	12·2 (11·6)
II	1·7	0·60	9·3 (11·0)
II	1·7	0·10	10·9 (11·7)
III	1·0	0·10	10·0 (11·7)

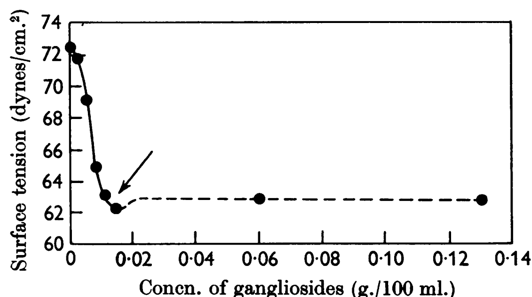

Fig. 3. Values of surface tension of the ganglioside preparation measured at 22° plotted against concentration. Details are given in the text. The break in the curve corresponding to the critical micelle concentration is marked with an arrow.

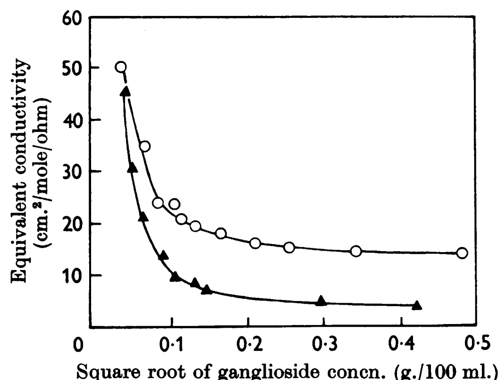

Fig. 4. Values of the equivalent conductivity of gangliosides in water measured at 0° plotted against the square root of concentration. ○, Acid form of gangliosides; ▲, sodium salt of gangliosides, prepared by neutralization with NaOH. Details are given in the text.

which micelles with hydrophilic groups outermost predominated were almost surface-inactive, and the monomers or small micelles present below the critical micelle concentration caused a lowering of surface tension of less than 10 dynes/cm.^{2}.

Electrical conductivities were measured at various concentrations for gangliosides in the acid form, prepared by passage of a solution of the preparation through mixed-bed ion-exchange resins, and for the sodium salt, prepared by neutralization with 0·1 N-sodium hydroxide. Equivalent conductivities were calculated from the observed specific conductivities and the equivalent weight for the preparation, namely 1520, and plotted against the square root of the concentration (Fig. 4). No well-defined breaks were apparent in the plots, although the equivalent conductivity did show a steep rise as the solutions were diluted beyond 0·01 %. A sharp break in the corresponding plot for detergents is coincident with the critical micelle concentration, the enhanced conductance at concentrations below this being ascribed to the increased number of units present, either monomers or small micelles each containing 3 or 4 units (McBain & Huff, 1948).

The magnitude of the surface charge on ganglioside micelles can be judged from the electrophoretic mobility measured in barbiturate buffer, pH 8·6 and I 0·1, namely $-8{\cdot}1 \times 10^{-5}$ cm.^{2}/sec./v (Table 1). This can be compared with the value of $-5{\cdot}2 \times 10^{-5}$ cm.^{2}/sec./v obtained under the same conditions for orosomucoid, which is the most acidic of the serum proteins and contains 12 % (w/w) of sialic acid (Schmid, 1953). On moving-boundary electrophoresis, gangliosides produced a single slightly asymmetric schlieren peak, closely similar to that observed in sedimentation.

Attempts to induce changes in the physical properties of the gangliosides. Ultrasonic irradiation of lecithin sols has a marked effect on the size of the micellar structures (Saunders *et al.* 1962). However, no change in the sedimentation coefficients could be detected after irradiation of ganglioside solutions of both high concentration (3 g./100 ml.) and low concentration (0·1 g./100 ml.) under similar conditions (Table 3).

Gangliosides were converted into the acid form during preparation by acidification of the final solution to pH 2·6 before freeze-drying. Removal of residual small ions by passage through mixed-bed ion-exchange resins resulted in an appreciable decrease in the conductivity of the solution, a fall in the already low phosphorus content and an increase in the polydispersity observed on sedimentation. A second component with a sedimentation coefficient of 7 s was produced which persisted after neutralization of the solution but which was no longer visible after dialysis against 0·1 M-sodium chloride.

Hydrolysis of gangliosides in 0·05 N-hydrochloric acid decreased the N-acetylneuraminic acid content by about 40 % and resulted in a changed pattern on thin-layer chromatography, the predominant species having 1 N-acetylneuraminic acid residue/sphingosine molecule (Fig. 1 b; Table 2). The electrophoretic mobility of the micelles formed by the hydrolysed material was $-5{\cdot}3 \times 10^{-5}$ cm.^{2}/sec./v, compared with a value of $-8{\cdot}1 \times 10^{-5}$ cm.^{2}/sec./v for the untreated gangliosides, a single boundary being observed. Sedimentation of a 1 % (w/v) solution of the hydrolysed gangliosides in NN-dimethylformamide gave an $S_{20,w}$ of 0·66 s. The equivalent weight measured by titration was 3010 and, as with the unhydrolysed material, only about 70 % of the carboxyl groups calculated from assay data were titrated.

Interactions with cations. The effect of additions of cations to gangliosides was studied by sedimentation and electrophoresis. Gangliosides in 0·2 M-sodium chloride and 0·2 M-calcium chloride gave $S_{20,w}$ values that were significantly greater than the mean value obtained in water (Table 3). Interactions with the basic dye, Cresyl Fast Violet, were examined, the dye being selected as an example of a large organic cation. The dye is insoluble in water but solutions of gangliosides were found to take the dye into solution, 10 mg. of ganglioside solubilizing 2 mg. of dye (mol.wt. 340). This represents an approximately equimolar mixture of ganglioside and dye. The ganglioside–dye complex was subjected to gel-filtration through Sephadex G-25, from which it was eluted in a volume similar to that required to elute untreated ganglioside micelles. No N-acetylneuraminic acid-containing material that had not bound the dye was detected in the eluate, so that all the gangliosides were assumed to have interacted with the dye. The ganglioside–dye complex had a greater sedimentation coefficient than the gangliosides alone, the difference being highly significant (Table 3), but the shape of the schlieren pattern was unchanged. In contrast, the ganglioside–dye complex had an electrophoretic mobility closely similar to that of gangliosides alone examined under the same conditions (Table 3).

Interactions with lysolecithin. The ability of gangliosides to form associations with other lipids was studied by using the water-soluble phospholipid, lysolecithin, derived from egg. Egg lysolecithin has been the subject of extensive physicochemical investigation (see Robinson, 1961); it has a micellar weight of 95 000 and an axial ratio of 3·5–4·0, being therefore spheroidal (Perrin, 1962). The mean sedimentation coefficient observed for 1 % (w/v) solutions of three separate preparations was 1·80 s (S.D. 0·04 s), the material sedimenting as a single boundary. Various proportions of aqueous 1 % (w/v) solutions of gangliosides and lysolecithin were mixed and examined in the ultracentrifuge. Sedimentation coefficients measured differed significantly from those of either of the individual lipids and varied in magnitude with their proportions in the mixture, the values ranging from 6·4 to 12·9 s.

Table 3. *Effects of various treatments on the sedimentation and electrophoretic behaviour of the ganglioside preparation*

Details are given in the text. The values in parentheses are values obtained for the untreated material.

Treatment	Concn. of ganglioside (g./100 ml.)	$S_{20,w}$ (s)	$10^5 u_{desc.}$* (cm.^{2}/sec./v)
(a) Ultrasonic irradiation of solutions in water	0·1	11·5 (11·6)	—
	3·0	8·4 (8·2)	—
(b) Sedimentation in salt solutions†			
(i) 0·2 M-NaCl	1·0	10·8 (10·5)	—
(ii) 0·2 M-$CaCl_2$	1·0	11·0 (10·5)	—
(c) Interaction with the basic dye, Cresyl Fast Violet‡	1·0	11·3 (10·5)	8·2 (8·1)
(d) Hydrolysis in 0·05 N-HCl for 30 min. at 80°§	1·0	—	5·3 (8·1)

* From electrophoresis in barbiturate buffer, pH 8·6 and I 0·1.

† Compared with values obtained in water.

‡ Dye was added until no more could be kept in solution by the gangliosides.

§ After hydrolysis, the sample was dialysed exhaustively against water, freeze-dried and finally dissolved in barbiturate buffer.

DISCUSSION

Properties of ganglioside molecules. The five components resolved on thin-layer chromatography of gangliosides on silicic acid in solvent systems with a low water content differed in their molar ratios of *N*-acetylneuraminic acid to sphingosine. This suggests that they are separated as individual molecules and that the separation depends on differences in chemical composition, species with lower R_F values having the higher molar ratios of *N*-acetylneuraminic acid to sphingosine. Some species were shown to contain 2 or more *N*-acetylneuraminic acid residues/molecule, confirming the observations of Rosenberg & Chargaff (1960) and Kuhn *et al.* (1961). The equivalent weight for the whole preparation, measured by titration, was 1520, which was close to the minimum molecular weight estimated from sedimentation in *NN*-dimethylformamide. Mild acid hydrolysis converted the gangliosides largely into the species containing 1 *N*-acetylneuraminic acid residue/sphingosine molecule. The equivalent weight of the resulting material was about twice that of the original preparation, but the sedimentation coefficient measured in *NN*-dimethylformamide was unchanged.

The electrophoretic mobility of the micelles found in aqueous solution was decreased in accordance with the lowered content of *N*-acetylneuraminic acid. A molecular weight of 1383 has been calculated from analytical data for gangliosides with 1 *N*-acetylneuraminic acid residue/molecule (Egge, 1961), so that a mixture of molecular species, some with 2 or more *N*-acetylneuraminic acid residues/molecule, would be expected to have an equivalent weight considerably less than 1383. However, titration data indicate that only about 70% of the carboxyl groups on the *N*-acetylneuraminic acid residues are titratable, so that equivalent weights obtained from titration data may be expected to be at variance with calculated values. It is not possible from the present data to establish which groups on the different ganglioside molecular species are available for titration.

Physical properties of ganglioside micelles. Aqueous solutions of gangliosides which contain micelles formed by the association of molecules give sedimentation patterns characteristic of polymolecular distributions, suggesting that a range of micellar sizes exists. Micellar weights calculated from sedimentation, diffusion and viscosity data ranged from 257000 to 446000. These values are somewhat greater than those measured for material prepared by other methods which have given weights of 180000 (Rosenberg & Chargaff, 1958) and 250000 (Folch *et al.* 1951; Trams & Lauter, 1962). Viscosity measurements indicate that the ganglioside micelles have an axial ratio of about 2, i.e. they are spheroidal in shape.

The fractions isolated from chromatograms formed micelles when taken up in water; these exhibited a smaller degree of polydispersity than those formed from the unfractionated material. Moreover, they had $S_{20,w}$ values that differed significantly from those observed for the original preparation when the concentration-dependence of sedimentation was taken into account. Thus the polydispersity of the ganglioside micelles, as observed in the ultracentrifuge, appears to be partly related to variations in the chemical structure of the constituent molecules.

From the micellar-weight calculations, the ganglioside micelle contains at least 200 monomers. On geometrical grounds severe limitations are placed on the number of molecules that can be packed into a spherical micelle. For molecules with small polar heads and large non-polar moieties, e.g. soaps, no more than 100 units can pack into a spherical micelle; so that for a larger number of units it has been necessary to postulate the existence of lamellar micelles (McBain, 1950) or sausage-shaped micelles, whose ends are spheres (Debye & Anacker, 1947). Ganglioside micelles assume a spheroidal shape, owing to the large polar moiety of the molecules compared with the small non-polar end which gives them a wedge shape. The size of the ganglioside micelle will depend on the length of the fatty acid chains in the constituent molecules, but, since stearic acid makes up over 80% of the fatty acid in ox-brain gangliosides (Trams *et al.* 1962), variations in micellar size from this source must be small. The polysaccharide moiety of the molecules, however, shows more variation. The sequence of sugars and amino sugars and the way in which the polysaccharide chain is branched must contribute appreciably to variations in the overall dimensions of the micelle by determining the mode of packing in the polar end of the molecule. From the high anodic electrophoretic mobility it is clear that the carboxyl groups of the *N*-acetylneuraminic acid residues of the molecules determine the charge on the micelles. In micelles containing molecules with 2 or more *N*-acetylneuraminic acid residues each, more than 1 of the constituent carboxyl groups/residue must, therefore, be near the surface of the micelle, hence contributing to the electrophoretic mobility and being available for titration.

Effect of cations on the properties of the micelles. The properties of ganglioside micelles appear to be influenced by changes in their association with small ions. The increased sedimentation coefficients observed in 0·2M-calcium chloride and 0·2M-sodium chloride compared with values obtained in water is interpreted as being due to binding at the anionic sites on the micelles rather than to an actual in-

crease in size due to more molecules associating in each micelle. Some of the increase may be due to repression of the primary charge effect in sedimentation, but this cannot here be distinguished from a binding phenomenon. The increased polydispersity of the ganglioside micelles after treatment of the preparation with ion-exchange resins to remove residual small ions was associated with the appearance of a slowly sedimenting component. This suggests that some rearrangement in the micelles takes place on removal of counter-ions, though some must remain firmly attached. It is unlikely that the change results primarily from removal of phospholipids solubilized by the ganglioside micelles, since dialysis against 0·1 M-sodium chloride caused the micelles to revert to their original form. Folch, Lees & Sloane-Stanley (1957*b*) have described the association of particular ions with particular lipids extracted from cerebral tissues by organic solvents. They did not, however, include gangliosides isolated after partition of extracts with dilute salt solutions. Any associations observed between lipids and ions may depend partly on the methods used for extraction and fractionation and not primarily on conditions in the intact tissue. However, the large contribution of the lipids as a whole to ion balance in cerebral tissues has been emphasized by McIlwain (1959), who suggested that this may be related to the role of both salts and lipids in cerebral functioning. The observation that cations influence the physical properties of isolated gangliosides in aqueous media may be related to their known activity in conditioning ion movements in slices of cerebral tissue (McIlwain *et al.* 1961).

Possible modes of interaction of gangliosides with other molecules. Gangliosides interact with other lipids; interaction with the water-soluble phospholipid, lysolecithin, led to the formation of units with sedimentation coefficients that differed significantly from those measured for the individual lipids. This suggests that mixed micelles were formed. Similar observations have been made with lysolecithin–lecithin mixtures that form predominantly asymmetric mixed micelles (Perrin, 1962). If it is assumed that the spheroidal shape of the lysolecithin and ganglioside micelles is determined by the large polar ends of the molecules, insertion of foreign lipids between the existing units in the micelles would be expected to result in a distortion of their shape, with a concomitant increase in the measured $S_{20,w}$. Lipid impurities in ganglioside preparations would be expected, therefore, to affect the size and shape of the micelles formed if the molecules became inserted between the ganglioside molecules. This may be reflected in the greater polydispersity of preparations containing large amounts of phospholipid impurity. High purity in lipid preparations to be examined by physicochemical methods is therefore important.

The interaction of gangliosides with proteins to form units of different physical dimensions (van Heyningen & Miller, 1961; Doery & North, 1961) is probably the result of van der Waals' forces. A mixture of tetanus toxin and ganglioside migrated on moving-boundary electrophoresis at about the same rate as did gangliosides alone, suggesting that whatever interaction occurs has little effect on the charge on the ganglioside micelles. Similarly, the complex formed between gangliosides and the basic protein, protamine, migrated to the same position on paper electrophoresis as did gangliosides alone (Booth, 1962). However, in this instance there is strong evidence that the interaction is predominantly ionic (Harris & Saifer, 1960*a*; Booth, 1962).

Interactions with basic dyes have given much information on the possible sites on the gangliosides available for interaction with other molecules. Harris & Saifer (1960*a*, *b*) studied the metachromasy of gangliosides in the presence of the basic dye, Toluidine Blue O, and showed that the metachromasy was suppressed by protamine. Cresyl Fast Violet is firmly attached to gangliosides; they also show positive metachromasy with this dye (van Heyningen & Miller, 1961). The attachment to ganglioside micelles resulted in an increase in the sedimentation coefficient without a change in the electrophoretic mobility (Table 3). Thus ionic interaction seems to be unlikely, and absorption into a region of the micelles with a low dielectric constant is postulated. Albers & Koval (1962) made a similar observation with berberine from variations in the fluorescence yield and fluorescence polarization of the dye in the presence of ganglioside. In contrast, the molecules of the basic dye, Acridine Orange, had a greater tendency to interact with each other at the colloidal interface.

Effect of size and structure of gangliosides in biological interactions. Interactions with ganglioside micelles may, therefore, involve the anionic groups or the hydrophobic moieties resulting in a change in their size or shape. The fixation of tetanus toxin (van Heyningen & Miller, 1961) and the restoration of the response of isolated cerebral tissues to electrical stimulation (McIlwain, 1961*b*) depend primarily on the availability of anionic groups. Removal of over 90% of the *N*-acetylneuraminic acid from gangliosides by acid hydrolysis almost completely destroys their ability to bring about restoration of response to electrical stimulation (H. McIlwain, unpublished work). The interaction with chlorpromazine, on the other hand, appears to be primarily by adsorption at the lipophilic region of the ganglioside micelle, since interaction is greater with material derived from gangliosides by

the removal of N-acetylneuraminic acid than with intact gangliosides (Albers & Koval, 1962).

The importance of the physical size of the gangliosides in biological interactions has not been fully realized, although Doery & North (1961) observed enhanced inactivation of staphylococcal toxin by material obtained from gangliosides by heating aqueous solutions which resulted in a loss of N-acetylneuraminic acid and a significant decrease in the sedimentation coefficient. Attempts to effect a decrease in the size of the ganglioside micelles by methods that did not involve hydrolytic cleavage of the molecules have been generally unsuccessful. Changes induced by treatment with ion-exchange resins were readily reversible. Ultrasonic irradiation of gangliosides solutions, with careful cooling to avoid local overheating, had no measurable effect on the sedimentation coefficient. This contrasts with the effect of ultrasonic treatment of concentrated egg-lecithin sols, in which microscopically visible myelinic structures of lecithin were broken down into micelles with micellar weights of about 5×10^6 (Saunders *et al.* 1962). The failure of the ganglioside micelles to be affected by cavitation, which is mainly responsible for breaking down structures in the liquid during ultrasonic irradiation, may be due to their comparatively small size.

A striking change in the size of the ganglioside units occurs as a result of dilution of aqueous solutions to very low concentrations, i.e. to below the critical micelle concentration of about 0·015 %, determined from surface-tension measurements. The rapid rise in the equivalent conductivity of solutions of gangliosides either in the free acid form or as sodium salts at low concentrations (Fig. 4) reflects an increase in the number of conducting units present, suggesting that dissociation commences in the region between 0·1 and 0·2 %. However, since no sharp break was apparent in the curves obtained, it is not possible to specify a true critical micelle concentration from these data. In studying the effect of concentration of ganglioside solutions on their ability to restore response to electrical excitation, McIlwain (1961*b*) observed that a concentration of 0·014 % gave nearly maximal restoration of response. Though this may represent the stoicheiometric amount of ganglioside for interaction with sites on the tissue, this concentration is near the measured critical micelle concentration, so that gangliosides may be most active in this system when in the form of individual molecules. In any event, at the critical micelle concentration gangliosides show the highest surface activity, i.e. any action at membrane surfaces in the tissue will be at a maximum.

The presence of both hydrophobic and hydrophilic groups in the ganglioside molecules is not only conducive to the formation of micelles in aqueous solutions but is also consistent with their occurrence in biological membrane structures. On examination of subcellular fractions of guinea-pig cerebral cortex, over half of the tissue ganglioside was obtained in the membrane-rich microsomal fractions (Wolfe, 1961; Wherrett & McIlwain, 1962). Further, their involvement in membrane phenomena, and specifically in conditioning ion movements, has been demonstrated (McIlwain *et al.* 1961). McIlwain (1962) suggested that gangliosides are, by their structure, not likely to constitute mobile ion carriers, but could constitute hydrophilic pathways across the lipid of the membrane. Determination of the precise role of gangliosides in the movement of ions across the neuronal membranes awaits further study of their relationship with other cerebral constituents involved in the process.

SUMMARY

1. Gangliosides have been isolated from ox-brain grey matter in a high state of purity containing only traces of phosphatides and cerebrosides. They were separated into at least five components containing N-acetylneuraminic acid on thin-layer chromatography.

2. The minimum molecular weight estimated from sedimentation in NN-dimethylformamide was 1500, which is close to the molecular weight calculated for gangliosides with 1 N-acetylneuraminic acid group/ceramide group. This also agrees well with the equivalent weight measured by titration.

3. In water gangliosides formed spheroidal micelles that gave micellar weights of 250000 to 450000 calculated from sedimentation, diffusion and viscosity data. The chemical constitution of the individual ganglioside fractions appeared to influence the size of the micelles formed in water.

4. A critical micelle concentration of about 0·015 % was observed from measurement of the surface tensions of aqueous solutions.

5. Subjecting gangliosides to mild acid hydrolysis removed half of the N-acetylneuraminic acid residues, resulting in a decrease in the charge on the micelles. The equivalent weight was increased to 3000.

6. The removal of associated small ions resulted in changes in the physical properties of the micelles. Ultrasonic treatment of ganglioside solutions had no measurable effect on their properties.

7. Gangliosides have been shown to interact with organic and inorganic cations and with other lipids.

8. The significance of the physical properties of gangliosides in biological interactions and in their postulated role in cerebral tissues is discussed.

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