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Gangliosides and Related Substances of Isolated Cerebral Tissues Examined in Relation to Tissue Excitability

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When cerebral tissues are kept in cold media they lose their normal metabolic response to applied electrical pulses (Marks & McIlwain, 1959). Response can be restored by incubating with tissue extracts and blood-plasma fractions, and the activity of such fractions has been correlated with their content of neuraminic acid derivatives (McIlwain, 1960*a*, 1961*a*). The most potent of these derivatives examined were the cerebral gangliosides, and it has therefore been considered that more might be learned about cerebral excitability by measuring the quantity of gangliosides and of related substances in the brain and in isolated cerebral tissues prepared and maintained in different fashions, including especially those kept in cold media and incubated with various additional substances.

The gangliosides (for nomenclature see McIlwain, 1961*c*) contain fatty acids, sphingosine, *N*-acetylgalactosamine and glucose as well as the *N*-acetylneuraminic acid by which they are most frequently determined (Svennerholm, 1957; Klenk & Gielen, 1960; Rosenberg & Chargaff, 1960; van Heyningen & Miller, 1961); they comprise more than one molecular species (Meltzer, 1959; Folch & Lees, 1959; van Heyningen & Miller, 1961). Cerebral tissues also contain derivatives of *N*-acylneuraminic acids (sialic acids) which either are not gangliosides or are further derivatives or complexes of gangliosides (Svennerholm, 1956; Rosenberg & Chargaff, 1958; and see below), and the particular distribution of gangliosides between aqueous salt solutions and non-aqueous solvents, investigated by Folch, Lees & Sloane-Stanley (1957), gives valuable means of obtaining additional specificity in methods for their determination. Long & Staples (1959) applied such distribution in a method for separating and determining the gangliosides, which forms the basis of that studied below. We have, however, given further attention to two aspects of the determination: (1) the completeness of extraction of the lipid-soluble neuraminic acid derivatives; (2) the use of an alternative spectrophotometric method for the measurement, namely the thiobarbiturate method of Warren (1959), as well as the ferric chloride–orcinol reaction as used by Long & Staples (1959).

EXPERIMENTAL

Tissues and metabolic experiments

Cerebral tissues. (*a*) For tissue rapidly fixed *in situ*, young rats were projected head first into approx. 400 ml. of liquid N_2 in a vacuum jar. After about 5 min., when vigorous bubbling had ceased, the animal was removed with cold tongs, wrapped in a cloth with its head projecting, fur was scraped from the head with a scalpel and the skull removed with a chisel also cooled in the liquid N_2. The brain was chiselled out from the frozen animal on to a pad of clean paper and quickly transferred to a cold glass-stoppered tube.

(*b*) Blocks of tissue removed at room temperature were obtained by stunning guinea pigs or rats by a blow on the back of the neck, exsanguinating, opening the skull, removing the brain and taking a sample with a scalpel within 3 min. of stunning the animal.

(*c*) Slices for incubation were cut from the brain removed as in (*b*), with a strip of razor blading and a recessed guide as described by McIlwain (1961*b*). The incubating medium (below) was used to lubricate the blade in cutting and slices (0·35 mm. in thickness) were obtained, floated free in a dish of medium, picked out with a wire rider, drained by repeated contact with a glass surface and weighed.

Media and incubation. All media contained (mM): NaCl, 134; KCl, 5·4; $CaCl_2$, 2·6; $MgSO_4$, 1·3; KH_2PO_4, 1·2; glucose, 10. In addition, the phosphate medium contained 10·4 mM-Na_2HPO_4 brought to pH 7·4 by HCl; the tris medium contained 25 mM-2-amino-2-hydroxymethylpropane-1:3-diol (brought to pH 7·4 by HCl) and the glycylglycine medium contained 30 mM-glycylglycine (brought to pH 7·4 by NaOH). Media were prepared from stock solutions immediately before use, oxygenated, distributed among conical manometric vessels of 15–20 ml. and any further additions made as indicated in individual experiments mentioned below. The tissues (described above) were added immediately after weighing; the vessels were equilibrated with O_2 and shaken at 37·5°.

In Expts. 6–8 of Table 5, electrical pulses were applied to slices incubated in silver-grid electrodes *H* in vessels *A* (Ayres & McIlwain, 1953; McIlwain, 1960*b*). Alternating pulses of exponential time–voltage relationship, time-constant 0·4 msec. and 10 v peak potential were applied at 100/sec.

Determination of ganglioside-like extractives

Extraction. Procedure *A*. Weighed specimens (80–100 mg.) were placed at 0° in homogenizer tubes, 150 mm. × 12 mm., provided with pestles ground smoothly to fit. From a $CHCl_3$–methanol mixture (2:1, v/v; also at 0°)

volumes (ml.) 19 times the weight of the tissue (g.) were pipetted into the tubes; the tissue was ground in the solvent and the mixture left for 3 min.

Extraction in the presence of added substances. In the experiments of Table 2, the additional substances were dissolved or suspended in 10 times their weight of water and added to the homogenizer tubes of procedure *A*. The weighed slices were then added and ground at 0° with the fluid for 3 min. or at intervals for 30 min. according to the experiment. The $CHCl_3$–methanol mixture, of volume 19 times the weight of tissue plus added water, was then pipetted into the tube and its contents were ground with the pestle. When the additional substance was suramin, used at 5 mg./100 mg. of tissue, the extraction is termed procedure *B*.

Extraction of fluids. From vessels after incubation, portions (2 ml.) of fluids were pipetted into 10 ml. beakers and evaporated to dryness over $CaCl_2$ *in vacuo*. The residue was rubbed up in 0·25 ml. of water or of a suramin solution, and ground with 19 vol. of $CHCl_3$–methanol (2:1, v/v).

Solvent partition. This procedure is based on those of Folch *et al.* (1957) and Long & Staples (1959). The $CHCl_3$–methanol tissue suspensions or fluid extracts were filtered by gravity through fluted circles of Whatman paper no. 43 (7 cm. diam.) into graduated 125 mm. × 16 ml. glass-stoppered tubes. The volumes of the filtrates were noted, 0·2 vol. of 0·1 M-KCl was added to each and the tubes were shaken thoroughly so that an emulsion was maintained for 10–15 min. The tubes were then centrifuged at 1200*g* for 3 min. and the upper layers (*a*) collected by Pasteur pipette. To the lower layers were added 0·4 vol. of $CHCl_3$–methanol–0·1 M-KCl (3:48:47, by vol.). The tubes were shaken and centrifuged as before, and their upper layers combined with layers (*a*). (A further extraction contributed less than 3% of additional chromogen, and was not normally performed. Filtration under the conditions specified removes non-ganglioside neuraminic acid-containing substances by absorption, a process to be reported in detail subsequently.)

Acylneuraminic acid determinations. (i) Bial's $FeCl_3$–orcinol reaction. This was close to the method of Böhm, Dauber & Baumeister (1954) as applied to cerebral tissues by Long & Staples (1959); Bial's orcinol reagent was made by dissolving 200 mg. of orcinol in 4 N-HCl, adding 1 ml. of 2% (w/v) $FeCl_3$ and making to 100 ml. with 4 N-HCl.

The upper layers after partition were evaporated to dryness in 125 mm. × 16 mm. tubes, in a stream of N_2 in a water bath at 80°. To the residues was added 2 ml. of 4 N-HCl, the tubes were shaken and 1 ml. was removed from each to a second set of similar tubes. One set of tubes was heated at 100° for 30 min. These constituted the control tubes; *N*-acetylneuraminic acid yields no purple but only a pale-yellow colour with the reagent after this treatment. To these tubes, cooled, and to the others, unheated, 1 ml. of orcinol reagent was added and the tubes were kept at 100° for 15 min. The tubes were then brought to room temperature in running water, 4 ml. of pentan-1-ol was added and the tubes were stoppered, shaken and centrifuged at 2000*g* for 3 min. Solution from the upper layers was taken by Pasteur pipette into 1 cm. cells of a Unicam SP. 500 spectrophotometer and *E* at 570 mμ measured. The extinctions, less their appropriate controls, were expressed as *N*-acetylneuraminic acid by comparison with a standard curve based on the values obtained by treating in the same way samples of 10–50 μg. of this substance.

(ii) Thiobarbituric acid reaction. A few modifications have been made in the method of Warren (1959), although the periodate, arsenite and thiobarbituric acid reagents which he describes were used.

The $CHCl_3$–methanol tissue extract was evaporated to dryness in N_2 (as described above) and the residue shaken with 1 ml. of water. Of this extract 0·18 ml. was pipetted into a 125 mm. × 16 mm. stoppered tube, 0·02 ml. of N-H_2SO_4 added and the tube with others placed in a bath at 80 ± 1° for 2 hr. To the cooled tubes 0·1 ml. of periodate reagent was added and, after 20 min. at room temperature, 1 ml. of arsenite reagent was added and the tubes were shaken; a yellow–brown colour formed transitorily and disappeared. Thiobarbituric acid reagent (3 ml.) was then added and the tubes were shaken and kept in a vigorously boiling-water bath for 15 min. They were cooled in running water for 5 min., 4·3 ml. of *cyclo*hexanone was added, the whole shaken, the mixture centrifuged, the upper layer transferred to the cell of the spectrophotometer and *E* measured at 549 mμ.

A study (D. A. Booth, in preparation; some experiments with Dr L. S. Wolfe) of the course of liberation and destruction of *N*-acetylneuraminic acid during treatment of ganglioside preparations with 0·1 N-H_2SO_4 indicated 2 hr. at 80° to give maximum values, which were, however, only 61·4 ± 0·6% of the *N*-acetylneuraminic acid of the specimen. This was found to apply also to the gangliosides of tissue specimens and values reported in this paper have been corrected for the incomplete recovery; comparative values by the orcinol and the thiobarbiturate methods are given in Table 4.

Materials

The ganglioside, clupein, salmine and *N*-acetylneuraminic acid specimens were those described by McIlwain (1961*a*). Suramin, the sodium salt of bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamine-4:6:8-trisulphonate)carbamide, was obtained from Imperial Chemical Industries Pharmaceutical Division and from Bayer Farbenfabrik A.G. Sodium iodate, $FeCl_3$ and Na_2SO_4 were analytical reagents and pentan-1-ol, orcinol, thiobarbituric acid, sodium arsenite and *cyclo*hexanone were laboratory reagents.

RESULTS

Change in gangliosides with loss of excitability

Though values for cerebral-ganglioside content are quoted, for example by Klenk (1955) and by Svennerholm (1957), no investigation appears to have been made of whether the substances are stable during the few minutes occupied in obtaining samples from the brain of experimental animals at ordinary temperatures. As several other substances undergo rapid changes during such sampling but can be estimated after the brain has been frozen *in situ* by dropping small animals into liquid nitrogen (see McIlwain, 1959), this procedure was applied to obtain samples for determination of gangliosides. Table 1 shows that tissue so fixed yielded almost the same quantity of gangliosides,

Table 1. *Ganglioside-like substances of tissues fixed* in situ *and kept* in vitro

Tissue extraction was by procedure *A* and determination by Bial's reaction (see Experimental section); mean values are followed by S.D. and number of determinations.

Tissue	Treatment before extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)
(1) Young rats,* whole brain	Frozen *in situ*	1·83±0·16 (6)
	Removed at room temperature	1·73±0·09 (4)
(2) Adult guinea pigs, cerebral cortex	Removed at room temperature	1·87±0·08 (10)
(3) As (2)	As (2), incubated for 40 min. in glucose–glycylglycine medium	1·72±0·08 (6)
(4) As (2)	As (3), salt soln. with 150 μg. of clupein sulphate/ml.	1·35±0·09 (6)
(5)† As (2)	Left at 0° in glucose–phosphate medium for 18 hr. and subsequently incubated for 40 min. in same medium	1·40±0·09 (6)
(6)† As (2)	As (5), but for 5 hr. at 0° in tris–glucose medium before incubation	1·53±0·05 (6)

* Recently weaned and weighing 70–90 g.
† In these experiments the media also were examined for ganglioside-like extractives, as described in the text.

determined according to Long & Staples (1959), as tissue removed at room temperature: values under the two conditions did not differ significantly. The cerebral cortex from adult guinea pigs gave ganglioside values similar to those of the whole brain of young rats.

Tissue samples 3–6 of Table 1 were prepared for incubation in a uniform fashion (see Experimental section), which involved weighing them after contact with aqueous media; absorption of fluid occurs and accounts for tissues 3 giving lower ganglioside values than tissues 2. Greater changes were, however, caused by exposing the tissues to the conditions refered to in the introduction, which caused loss of their excitability. Thus the presence of protamine during incubation, or prior exposure of the tissues in cold media, caused diminution of 12–22% in the ganglioside extracted (tissues 4–6, Table 1). In two of these experiments, attempts were made to detect gangliosides in the media in which the tissues had been exposed or incubated. Although gangliosides added to such media could be estimated with an accuracy equivalent to the detection of 7 μm-moles of *N*-acetylneuraminic acid/100 mg. of tissue, none could be detected in the media when 35 μm-moles/100 mg. had been lost from the tissue.

Media in which cerebral slices had been incubated with protamine were examined semi-quantitatively for the presence of protamine by adding picric acid or fluorescein as described by McIlwain (1961*a*). These reagents form insoluble salts with protamine in the concentrations added before incubation, and showed an apparent loss of protamine from the incubating fluid. It was therefore thought that a protamine–ganglioside complex might have formed in the tissue itself, for basic proteins had proved to become attached to ganglioside-rich subcellular fractions of ground cerebral tissues (Wolfe & McIlwain, 1961). The attachment of protamine to slices has since been demonstrated quantitatively (Thomson & McIlwain, 1961). Presumably, therefore, this attachment was associated with either a breakdown of cerebral gangliosides, or with diminution in their extractability.

Binding of ganglioside-like substances in cerebral tissues; a new reagent for their extraction

Reagents which combined with basic proteins were therefore examined as aids to the extraction of ganglioside-like substances. The extraction of Folch *et al.* (1957) and Long & Staples (1959) involves grinding or blending tissues with chloroform–methanol (2:1, v/v). In testing the effect of added substances, these were dissolved or suspended in a small volume of water and the tissue was ground first with the added reagents; the mixture was then treated as usual with chloroform–methanol. Fluorescein, referred to above, was found unsuitable as a reagent, for it interfered with the subsequent determination of gangliosides. A number of acidic substances of relatively high molecular weight were being examined for their ability to restore tissue excitability (McIlwain, 1960*a*, 1961*a* and unpublished work) and several of these substances were tested as possible agents in release of gangliosides in normal and in protamine-treated tissues (Table 2).

In normal tissues freshly removed from guinea pigs, less ganglioside was obtained in the presence of polygalacturonic acid or of hydrochloric acid. Chondroitin sulphate and trypan red, though forming precipitates with protamine, did not in-

Table 2. *Acidic compounds and extraction of ganglioside-like substances*

Tissues were ground first with any added substances, and then with $CHCl_3$–methanol, as described in the Experimental section. Mean values are followed by S.D. and number of experiments, except when only two or three determinations were made.

Tissue	Added substance (quantity/100 mg. of tissue)	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)
(1) Guinea-pig cerebral cortex removed at room temperature	None	1·74±0·08 (11)
	N-HCl, 0·1 ml.	0·81
	Polygalacturonic acid, 10 mg.	1·11±0·12 (4)
	Chondroitin sulphate, 2 mg.	1·63±0·05 (4)
	Trypan red, 2 mg.	1·71±0·11 (4)
	Suramin, 2 mg.	2·07±0·11 (4)
	Suramin, 5 mg.	2·34±0·06 (4)
	Suramin, 5 mg.*	2·36
(2) Young rats, whole brain frozen *in situ*	None	1·83±0·16 (6)
	Suramin, 1·7–2·3 mg.	2·28±0·16 (6)

* Tissue was left for 30 min. after grinding with suramin; in all other cases tissue was left for 2–3 min.

crease ganglioside extraction. Increased extraction was, however, obtained with an antitrypanosomal agent, suramin: the sodium salt of bis-(*m*-amino-benzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamine-4:6:8-trisulphonate)carbamide. Suramin gave increased extraction of ganglioside-like substances from the brain fixed *in situ* with liquid nitrogen, as well as from tissues removed promptly at room temperature; 2–3 min. exposure to suramin was adequate (Table 2). The increased ganglioside readings did not appear to be artifactual: in the absence of tissue, the full procedure of extraction, solvent distribution and determination of *N*-acetylneuraminic acid could be carried through without the appearance of 'gangliosides' if none had been added, and if they, but no tissue, had been added the same recovery (95 ± 3%) was obtained with or without suramin. The quantity of suramin needed was concluded from the experiments of Fig. 1 to be above 3 mg./100 mg. of tissue, and 5 mg./100 mg. was used in subsequent experiments unless stated otherwise.

Recovery of added gangliosides and properties of the additional suramin-extracted material. Recovery of gangliosides added to mixtures containing cerebral tissues immediately before their extraction has now been re-appraised. Table 3 (Expts. 1 and 2) shows that under the conditions of Long & Staples (1959) recovery, though good, was significantly below 100%. In these experiments the quantity of added gangliosides was commensurate with the quantity native to the tissue. With added suramin, not only were higher values obtained for tissue gangliosides but, also, the recovery of added material was increased.

To learn more about the additional material extracted in the presence of suramin, assays for *N*-acetylneuraminic acid were made not only with Bial's reagent, which formed the basis of the

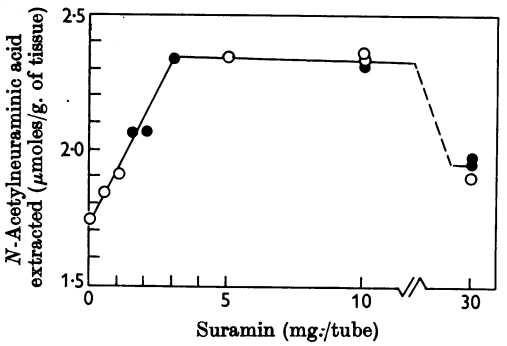

Fig. 1. Ganglioside-like material extracted from guinea-pig cerebral cortex in the presence of different quantities of suramin. Samples of 80–100 mg. of cortex were ground with suramin and $CHCl_3$–methanol as described in the Experimental section; determination was by Bial's reaction. ○ and ●, Different samples of suramin.

assays already discussed, but also with a thiobarbiturate method based on that of Warren (1959). An examination of the thiobarbiturate method specifically in relation to gangliosides, and in comparison with Bial's reagent, is being made (see Experimental section). Table 4 shows that both methods give a similar picture of increased extraction in the presence of suramin, and both were applied to see whether *N*-acetylneuraminic acid-containing material, not extracted by chloroform–methanol alone, could subsequently be obtained by adding suramin to the tissue residue, grinding and re-extracting. Any further extraction was small. As the brain is reported to contain protein-bound *N*-acetylneuraminic acid (Svennerholm, 1956), a preliminary examination has been made by paper electrophoresis (D. A. Booth, unpublished work) to detect such substances in the present extracts. Any additional amino acid-

Table 3. *Recovery of gangliosides added to tissue*

The quantities of gangliosides noted, in 50 or 100 µl. of water, were added to one of a duplicate set of tubes; those of Expt. 3 also contained suramin (2 mg.). Weighed portions of guinea-pig cerebral cortex (approx. 80 mg.) were then added to all tubes, followed by $CHCl_3$–methanol (2:1, v/v). After grinding, the tubes were left for 15 min. and the determinations completed by Bial's reaction as described in the Experimental section.

Expt. no.	*N*-Acetylneuraminic acid (µmole/tube) Added	Found	Calc. from controls, as present in the tissue taken	Recovery (% of that added as ganglioside)
1	0·084	0·233	0·155	93±7 (4)
2	0·168	0·294	0·139	92±5 (4)
3	0·084	0·275	0·188	103±6 (4)

Table 4. *Comparison of two ways of applying suramin; two assay methods*

Blocks of about 1 g. from guinea-pig cerebral hemispheres were placed in tubes, some of which (Expt. 1) contained 20 mg. of suramin in 0·2 ml. of water. The tubes of Expt. 2 (*a*) received $CHCl_3$–methanol (2:1, v/v), 19 times the volume of the tissue, and those of Expt. 1, $CHCl_3$–methanol 19 times the volume of tissue plus added water. After grinding and centrifuging, determinations were completed as described in the Experimental section. In addition, the residue from the extraction of Expt. 2 (*a*) was ground with suramin (20 mg. in 0·2 ml. of water) and the mixture ground with $CHCl_3$–methanol as in Expt. 1.

Expt. no.	Extraction	Ganglioside-like extractives (µmoles of *N*-acetylneuraminic acid/g. of tissue) Bial's reaction	Thiobarbituric acid method
1	Suramin present initially	2·22	2·20
2	(*a*) Initial extraction without suramin	1·86	1·84
	(*b*) Residue re-extracted in the presence of suramin	No detected	0·19

containing material in extracts made in the presence of suramin was not, however, associated with *N*-acetylneuraminic acid.

Gangliosides during loss and recovery of tissue excitability

The change in gangliosides previously observed to accompany loss of excitability has now been re-investigated with the new extraction method. In Table 5, tissue treated according to procedures 3, 4 and 5 lost excitability and all are now seen to yield less gangliosides in the presence as well as in the absence of suramin. Comparison of tissues incubated without and with protamine is particularly instructive, as these are brief procedures; a proportion of the gangliosides has, however, become inextractable.

The tissue content of ganglioside-like substances has been determined also under conditions which led to recovery of excitability. After keeping in cold media, tissues were incubated with added gangliosides (Table 6). This led to increase in their ganglioside content, whereas tissues which had not been previously treated in the cold picked up much less gangliosides on incubation. In appraising these values, it is necessary to consider how much of the added gangliosides could have become associated with the tissue in extracellular fluids. At the end of the experiments slices were drained from excess of fluid but could have up to 80 mg. of extracellular fluid/100 mg. original fresh wt. of tissue. Supposing this to be completely accessible to the ganglioside solution containing 0·5 mg./3·5 ml., or 0·12 µmole of *N*-acetylneuraminic acid/ml., the associated ganglioside could contribute some 0·1 µmole of *N*-acetylneuraminic acid/g. of tissue. Thus the small increase in ganglioside content in the untreated tissue of Table 6 could be attached in this relatively mechanical fashion. After keeping at 0°, however, the tissue takes up six times this quantity of ganglioside. Treatment of the tissue with protamine also increased its ability to take up ganglioside. Suramin released additional ganglioside-like material from all tissues.

The various associations observed between tissue gangliosides and excitability led to examination of whether electrical pulses themselves changed the tissue content of ganglioside-like substances (Table 5, Expts. 6–8). No change was found, however, in the presence or absence of protamine.

Table 5. *Suramin-released gangliosides of incubated tissues*

Guinea-pig cerebral cortex was used as slices of 80–100 mg. and extracted without and with suramin, by procedures *A* and *B* of the Experimental section; determination was by Bial's reaction.

Treatment of tissue before extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)	
	Without suramin	With suramin
(1) None	1·74±0·08 (11)	2·34±0·06 (4)
(2) Incubated for 40 min., glycylglycine–glucose medium	1·72±0·08 (6)	2·35±0·09 (6)
(3) As (2), medium with 150 μg. of protamine SO_4/ml.	1·35±0·09 (6)	2·04±0·09 (4)
(4) Kept for 5 hr. at 0° in tris–glucose medium, then incubated at 37° for 40 min.	1·53±0·05 (6)	1·96±0·04 (6)
(5) Kept for 18 hr. at 0° in phosphate–glucose medium, then incubated at 37° for 40 min.	1·40±0·09 (6)	2·01±0·06 (6)
(6) Incubated for 80 min. in glycylglycine–glucose medium, with electrical pulses during the final 40 min.	1·86±0·07 (4)	2·31±0·06 (10)
(7) As (6), medium with 150 μg. of protamine SO_4/ml.	1·42±0·04 (4)	2·02±0·08 (4)
(8) As (6), medium with 500 μg. of protamine SO_4/ml.	1·51±0·08 (4)	2·09±0·06 (4)

Table 6. *Normal and treated tissues incubated with gangliosides*

Guinea-pig cerebral cortex, sliced, was incubated for 40 min. in the oxygenated media in manometric vessels. Gangliosides when added were at 143 μg./ml. in the incubation medium and protamine (clupein sulphate) at 200 μg./ml. Extraction of the tissue was by method *A* (no suramin) or *B* (with suramin). Values are the mean of two or three determinations except when followed by S.D. and number of determinations.

Treatment of tissue before extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)		
	No suramin	Suramin (2 mg.)	Suramin (5 mg.)
(1) Incubated, glycylglycine medium	1·84	2·07	2·35±0·06 (6)
(2) As (1), medium with gangliosides added after 15 min.	1·93	2·17	—
(3) Incubated, glycylglycine medium with protamine	1·42	2·04	—
(4) As (3), medium with protamine and gangliosides*	1·59±0·03 (4)	2·13±0·09 (4)	2·29±0·10 (4)
(5) At 0°, 18 hr. in phosphate medium, then incubated	1·31	1·94	—
(6) As (5), gangliosides added before incubation	1·89±0·08 (4)	2·13±0·08 (4)	2·27±0·07 (4)

* In one set of experiments, gangliosides were added from a side arm after incubation for 15 min., as in treatment (2). In another set, the protamine-containing medium was removed after 15 min. and replaced by fresh medium containing gangliosides and no protamine. The values were closely similar.

DISCUSSION

The evidence presented above gives further reasons for considering gangliosides to be involved in the electrical excitability of cerebral tissues. Previous studies (see introduction) showed their potency in restoring excitability to tissues (i) kept in cold media, and (ii) treated with basic proteins. Now (iii) the tissues kept in cold media have been found to yield less gangliosides to chloroform–methanol, as also (iv) did those treated with basic proteins. Also (v) addition of ganglioside to tissues kept in cold media led to an increase in their ganglioside content which did not occur with normal tissues; and (vi) suramin, which increased the response of tissues treated as in (i) and (ii), permitted the extraction from such tissues by chloroform–methanol of a further quantity of ganglioside-like material. The connexion of gangliosides with processes of excitation is, however, of a different type from those exhibited by, for example, glucose, which is consumed, and phosphocreatine, which undergoes a reversible change in quantity, during a few seconds' stimulation. Change was not detected in gangliosides during removal of the brain at room temperature, nor during electrical excitation *in vitro*.

Accepting the conclusion (Thomson & McIlwain, 1961) that interactions between protamine, suramin and tissue gangliosides show neuraminic acid of gangliosides to contribute negative surface groups concerned in tissue excitation, it would now appear that not all neuraminic acid residues of ganglioside-like substances are in this position. Not all such substances were rendered inextractable by protamine, and suramin released a further quantity

from normal, excitable, tissue. Investigation of whether this behaviour is due to chemical heterogeneity in gangliosides or to differences in their location in the tissue, or to both factors, is in progress. The ready combination of suramin with proteins (Town, Wills, Wilson & Wormall, 1950) suggests that the gangliosides released by suramin may be normally linked in some distinctive fashion to tissue protein. The subcellular fractions of cerebral cortex which carry gangliosides (Wolfe & McIlwain, 1961; Wolfe, 1961) carry also an adenosine triphosphatase which is activated by Na^+ and K^+ ions (J. Järnefelt, personal communication; D. H. Deul & H. McIlwain, in preparation). This enzyme is inhibited by suramin (D. H. Deul & H. McIlwain, in preparation). Gangliosides restore active Na^+ and K^+ ion transport to tissues in which this has been inhibited by protamine (Cummins & McIlwain, 1961; Woodman & McIlwain, 1961). These, with the present findings, have been incorporated into a mechanism of ion transport which is outlined by McIlwain (1960*c*).

SUMMARY

1. *N*-Acylneuraminic acid derivatives have been determined by the orcinol–ferric chloride reaction in cerebral tissues, after extraction and solvent partition by methods which yield ganglioside preparations. The resulting values for ganglioside-like substances are expressed by reference to synthetic *N*-acetylneuraminic acid. Tissues frozen *in situ* gave values of 1·8 μmoles/g., close to those of tissue dissected at room temperature.

2. Incubation in oxygenated glucose media had little effect on these values, but diminution to 1·3–1·5 μmoles/g. was found after incubating with protamine or after keeping in cold media before incubation. These are conditions under which the tissue's metabolic response to electrical pulses is lost.

3. Further quantities of ganglioside-like substances could be extracted from tissues in the presence of suramin. A number of other substances were inactive in this respect; suramin increases response by the tissue.

4. Suramin also increased the yield of ganglioside-like substances when these were estimated by an independent method which depended on hydrolysis and reaction with periodate, followed by thiobarbituric acid.

5. The total ganglioside-like substances, which included those extracted in the presence of suramin, also diminished under the conditions of paragraph 2 above. Subsequent incubation with gangliosides under conditions allowing restoration of response to pulses, increased the ganglioside extractable from the tissue. Incubation with gangliosides did not have a comparable effect on normal tissue.

6. The findings indicate heterogeneity in the tissue gangliosides and the association of at least one component with the tissue's ability to respond to pulses.

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