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Characterization of Naturally Occurring Materials which Restore Excitability to Isolated Cerebral Tissues

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Cerebral tissues kept in cold media lose their respiratory and other responses to applied electrical pulses (Marks & McIlwain, 1959). The following properties show that the unresponsive state induced in this way is relatively well defined: (1) several metabolic characteristics of the tissue, in the absence of pulses, were little affected when the metabolic response of the tissue to pulses was depressed; (2) unresponsive states could be induced in the tissue by keeping it under other adverse conditions, but differed from that induced in cold media in several ways, including the type of substances which contributed to restoring response (McIlwain & Gore, 1953; McIlwain, 1956); (3) a variety of agents added to the tissue while it was kept in cold media did not affect the loss of its excitability.

By using as a test system the tissue made inexcitable in cold media, materials which could restore excitability were shown to be present in blood plasma and in the brain. The following properties indicated marked specificity in the action of restoring agents: (1) the active preparations were without appreciable effect on the respiration of untreated cerebral tissues, whether or not pulses were applied to them; (2) the restoring agents had little or no effect on the respiration of the treated tissue in the absence of applied electrical pulses; (3) the effects of the restoring agents were not shared by extracts of muscle or by some 30 known compounds or groups of compounds. An initial exploration of the chemical properties of the active material in blood plasma was described by Marks & McIlwain (1959) and by McIlwain (1959*a*; 1960*a*); it is the further characterization of this material, and of an active substance from the brain, which are now to be described.

During the initial stages of this study, and while the active substances of blood plasma were still uncharacterized, preparations from plasma were used to study the nature of the change which occurred when the tissue was kept in cold media. It was concluded that the unresponsive state so engendered was caused by migration of the tissue's histone. Further evidence regarding this is given in an accompanying paper (Wolfe & McIlwain, 1961). An analogous unresponsive state could be induced in normal cerebral tissues by adding histones and related compounds, and this unresponsive state also has therefore been studied in the present investigation.

EXPERIMENTAL

Tissue metabolism and response to pulses

The cerebral cortex from guinea pigs was used and its response studied in the following experimental arrangements:

Exposure at 0°. Tissues were prepared and exposed in cold media as specified by Marks & McIlwain (1959), with conditions *C* in which cortical slices were kept at 0° for 5 hr. in glucose-containing media buffered with 2-amino-2-hydroxymethylpropane-1:3-diol (tris). After being kept at 0° the tissue specimens (inner slices with two cut surfaces, fresh wt. about 45 mg.) were mounted in silver-grid electrodes and placed in vessels *E* (Ayres & McIlwain, 1953) containing 3·5 ml. of glucose–tris medium. In some of the vessels the medium received the substances which were being examined as agents restoring excitability. Respiratory rate was measured during 40–45 min. at 37·5°, after which alternating condenser pulses of peak potential 10 v and 0·4 msec. time-constant were applied at 100/sec. and measurement of respiration was continued for a further 40–60 min. At the end of an experiment the electrodes with their tissues were removed promptly from the vessels.

Inhibition by proteins. The unresponsive state induced by basic proteins was examined as specified by McIlwain (1959*a*): immediately after weighing, the tissue samples were placed in the electrodes of the apparatus described above, immersed in glycylglycine-buffered media, and the respiration was measured at 37·5° before and during the application of electrical pulses. The following three types of experiment were involved:

(*a*) The inhibitory potencies of different proteins and peptides were compared by including them in a range of concentrations in the experimental media into which the slices were placed.

(*b*) The ability of added substances to restore excitability to tissue exposed to basic proteins was examined under the conditions of McIlwain (1959*a*, Tables 4 and 5). This involved incubating tissue specimens in electrodes in a series of vessels, some with and some without the basic proteins, for 15 min. The vessels were then taken from the thermostat and from their manometers, and the media removed from the vessels with Pasteur pipettes. The tissues remained in their electrodes in the vessels during this procedure, and the tissues, electrodes and vessels were further washed with 1 ml. of medium which in all the vessels was without

inhibitor. Medium alone, or media with the substances being examined as agents restoring the excitability, were then pipetted into the vessels. The vessels were replaced on their manometers, re-equilibrated with oxygen, and respiration was measured before and during the application of pulses as above.

(*c*) For examining the capabilities of added substances in preventing the establishment of an inhibitory condition in the presence of basic proteins, media containing the protein were added to a series of manometric vessels. To some of the vessels were then added the substances being examined as antagonists. About 15 min. later, tissues in their electrodes were placed in the vessels and the experiments completed as above. In a few instances (Table 4) application of electrical pulses was replaced by the addition of 0·15 ml. of M-KCl from the side arm of manometric vessels, to 3·5 ml. of medium.

Control experiments. The following experiments were carried out to ensure that the changes in gas pressure measured manometrically were due to tissue respiration. Pulses of the parameters described above were applied to vessels containing electrodes, media and the added compounds under examination, but no tissue. No progressive pressure changes were observed; these experiments included the gangliosides (0·4 mg./ml.) to control the experiments of Tables 2 to 4, and gangliosides (0·4 mg./ml.) plus protamine (0·15 mg./ml.) to control those of Tables 3 and 4. Also, as is noted in Table 3, gangliosides were examined in the presence of normal tissue (i.e. not kept at 0°) and were not found to increase its respiratory response to pulses.

Materials and analyses

Ox-blood plasma fractions and cerebral extracts were those described by Marks & McIlwain (1959). Basic proteins included those described by McIlwain (1959*a*), the protamine being clupein sulphate (L. Light and Co. Ltd.) unless otherwise stated. Analysis of the specimen was as follows (Weiler and Strauss, Oxford): total N, 24·6; total S, 5·78%. These values are very close to those of Carroll, Callanan & Saroff (1959) for purified salmine and, as the protamines have no sulphur-containing amino acids, the values imply an equivalent weight of 277 for the specimen used (cf. Table 6). For a freshly made specimen of calf-thymus histone sulphate, I am indebted to Dr L. S. Wolfe; it gave: total N, 13·85; total S, 4·31%. On a similar basis (see Crampton, Stein & Moore, 1957) this implies an equiv. wt. of 371. The specimen of poly-L-lysine hydrochloride, degree of polymerization approx. 30, was from Mann Research Laboratories Inc., New York. A specimen of the sialomucopolysaccharide, isolated from human ovarian-cyst fluid by Pusztai & Morgan (1960), was kindly given by Professor W. T. J. Morgan.

Gangliosides. Two preparations (I) of ox-brain gangliosides were made largely according to Folch, Lees & Sloane-Stanley (1957), the choice of some conditions being that of Long & Staples (1959). Two ox brains were freshly obtained from a slaughter house and cerebral cortex (50 g.) was taken with relatively little admixture with white matter, by snipping off portions of about 0·4 g. with scissors. This was blended in a top-drive macerator with the chloroform–methanol mixture (950 ml.) for 2 min. and the suspension filtered by gravity through fluted Whatman no. 43 papers. The filtrate was distributed among six 200 ml. centrifuge bottles and one-sixth of its vol. of 0·1 M-KCl added to each. The bottles were stoppered and shaken well, so that an emulsion was maintained for 15 min., and were then centrifuged at 1500 *g* for 20 min. The upper layers were collected by suction for the main ganglioside preparations. Two subsequent extractions (*e*) of the lower layers were made but the materials obtained have not been used in the present study. Each upper phase was dialysed in Visking tubing (diam. 1 in.) tied to form sacs which were immersed in five successive batches of 16 l. of distilled water at 0–2°; they remained with each batch of water for approx. 12 hr. The water was contained in cylinders 52 cm. high and agitated with a Vibromix stirrer (Shandon Scientific Co. Ltd., London; from A. G. für Chemie-Apparatebau, Zürich), which is very effective in aiding dialysis as material inside the sacs is also agitated by the 50 cyc./sec. vibration. After dialysis the sac contents were evaporated at a few mm. Hg (bath temp. 60°, vapour temp. 20°) to about 8 ml., and the slightly yellowish solution was washed into a Visking sac (diam. $\frac{1}{4}$ in.) and then dialysed as before for 16 hr. The sac contents were filtered into a crystallizing dish and this was placed above $CaCl_2$ in a desiccator, which was evacuated. Fine white strands separated in about half an hour, before the solution had evaporated, and after some hours were left as a light white fibrous solid, yield 93 mg. The two extractions (*e*), when treated in the same way, yielded yellow scales 0·1 and 0·03 of the weight of the main batch.

A further ganglioside preparation (II) was given by Dr C. Long and was the 'partially purified ox-brain strandin' of Long & Staples (1959), having been prepared by Dr G. H. Sloane-Stanley. For a preparation (III) made according to Folch, Arsove & Meath's (1951) procedure for strandin, I am indebted to Dr S. Balakrishnan. A preparation (IV), purified by chromatography on silicic acid according to W. E. van Heyningen & P. M. Miller (unpublished work) was given by Dr W. E. van Heyningen.

Neuraminic acid content. Determinations were carried out with Bial's orcinol–$FeCl_3$–HCl reagent; for the synthetic *N*-acetylneuraminic acid (Carroll & Cornforth, 1960) used as standard, I am indebted to Miss Patricia Carroll. In plasma fractions the reagent was used as described by Böhm, Dauber & Baumeister (1954), with samples usually of 5 mg. of the fractions. In cerebral preparations the method of Long & Staples (1959) was used: a sample equivalent to 200 mg. of brain was divided into two parts, placed in 10 ml. tubes in a water bath at 80–100° and evaporated to dryness in a stream of N_2. To each tube, after cooling, was added 1 ml. of 4 N-HCl and one tube was heated at 100° for 30 min. and cooled. To each, 1 ml. of Bial's reagent prepared in 4 N-HCl was added, and both tubes were heated for 15 min. at 100°. Extraction with pentan-1-ol, photometric reading and preparation of standards (10–60 μg. of *N*-acetylneuraminic acid) followed the description of Long & Staples (1959).

The ganglioside preparations showed the following content of neuraminic acid derivatives, expressed as the *N*-acetyl compound: I, main batches 24 and 26·5%; II, 22%; III, 10%; IV, 28·5% (quoted by Dr van Heyningen: 0·928 μmole/mg. or 28·7%; and 0·308 μmole of *N*-acetylgalactosamine/mg.). Similarly expressed, the sialomucopolysaccharide contained 17% of neuraminic acid derivatives. Their purification has not been attempted in the present study, but the following data are relevant. The ganglioside preparations of Svennerholm (1956) and of Rosenberg & Chargaff (1958) contained neuraminic acid

equivalent to about 26% of the *N*-acetyl derivative. Meltzer (1958), after extensive fractionation, obtained material containing 33% of 'sialic acid' (the term is a generic one for the various acyl derivatives of neuraminic acid). Svennerholm (1956) obtained the preparation quoted above from cerebral tissues dehydrated with acetone before extracting with non-aqueous solvents; when, as in the present preparation I, extraction was of fresh brain, the product yielded amino acids on hydrolysis. Le Baron & Folch (1957) obtained a similar preparation which yielded a high proportion of arginine among the amino acids obtained on hydrolysis; the formation of complexes, reported below, may be relevant to this result.

Precipitation reactions

The data of Table 5 were obtained by mixing at room temperature (about 16°) the basic proteins and the neuraminic acid derivatives, each dissolved in one of the media already quoted or in 33 mM-glycylglycine brought to pH 7·4 by NaOH. Mixtures were made in volumes of 0·2–0·3 ml. and at dilution intervals of approx. 30%, and they were observed at intervals for about 1 hr. Picric acid was used as a reagent as a saturated aqueous solution of 1·4 mg./ml. or 6·1 mM, and sodium fluorescein at a concentration of 10 mg./ml. or 26·4 mM. They were added to give concentrations in the solutions being tested of 0·6 and 1·66 mM, and the solutions were observed at intervals for some hours. With protamine only, at about 100 μg./ml., picric acid yielded an immediate cloudiness, becoming a flocculent brown precipitate in a few minutes. With histone at a concentration of about 300 μg./ml., this occurred rather more slowly. Fluorescein gave bulkier, darker precipitates with both. Neither picric acid nor sodium fluorescein appeared to interact with the gangliosides or with the sialomucopolysaccharide.

RESULTS

Properties of blood-plasma fractions

Blood plasma and serum from ox and man were previously found of similar activity in restoring excitability to cerebral tissues which had been kept in media at 0°; the active material was non-dialysable, was precipitated by acetone or by ammonium sulphate, and was partly inactivated on heating (Marks & McIlwain, 1959). In the separation of ox plasma with ethanol at defined pH, according to Cohn *et al.* (1946), fractions IV-1 and IV-4 were found markedly more active than others in restoring excitability. A number of qualitative tests have now been applied to these and to the less active fractions. Molisch's reaction for carbohydrates (α-naphthol and a lower layer of conc. sulphuric acid) yielded with the active fractions not only the purple of the test, but also a distinct and intense brown layer above the purple one. A similar, extraneous brown colour was also given in the orcinol–sulphuric acid reaction for protein-bound hexose, carried out according to Winzler (1955), and the brown colour in both tests was then found to be given by the addition of sulphuric acid alone. It was given to a much lesser degree, if at all, by similar quantities of the other plasma fractions. At this point the 'browning reaction' of strandin was recollected. This reaction, observed by Folch *et al.* (1951), was later shown to be due to neuraminic acid derivatives (Rosenberg & Chargaff, 1958). Neuraminic acid derivatives occur in blood plasma in certain of the mucoproteins, and fraction IV-4 was found by Surgenor, Strong, Taylor, Gordon & Gibson (1949) to be rich in mucoproteins, though the type of mucoprotein was not then specified.

These observations prompted the determination of neuraminic acid derivatives in all the plasma fractions previously tested in restoring tissue excitability. Markedly higher contents of neuraminic acid derivatives were found in fractions IV-1 and IV-4 (Table 1). The fractions are precipitated in the order

Table 1. *N-Acetylneuraminic acid of ox-plasma fractions*

The fractions (Marks & McIlwain, 1959) were prepared by the method of Cohn *et al.* (1946), whose enumeration is followed. Determinations were according to Böhm, Dauber & Baumeister (1954) and the values quoted are the average of duplicate or triplicate results agreeing within 5%.

Fraction	*N*-Acetylneuraminic acid (mg./g. dry wt.)
I	4·0
II	2·4
III-1	6·6
III-2	6·8
IV-1	19
IV-4, batch C 183	23·2
IV-4, batch BP 45	23·4
V	2·4
Albumin, crystalline	< 0·02

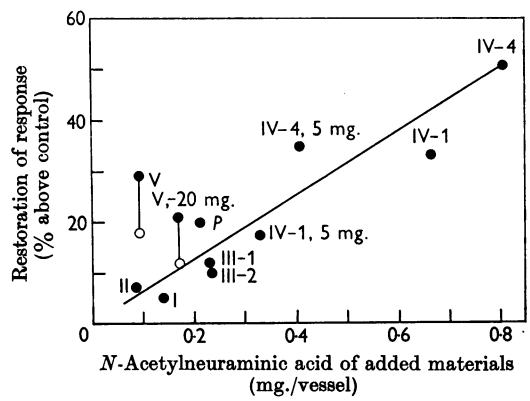

Fig. 1. Restoration of response by blood-plasma fractions in relation to their content of *N*-acetylneuraminic acid. Response is the respiratory response to electrical pulses of a 45 mg. slice of guinea-pig cerebral cortex in 3·5 ml. of fluid, determined as described in the Experimental section. *N*-Acetylneuraminic acid is the content (see Table 1) of material added to the 3·5 mg. of fluid, the additions being of 10 mg. dry wt. of material/ml. of fluid, except when indicated as being 5 or 20 mg./ml. *P*, Plasma; I–V, fractions of Table 1.

listed in Table 1, which thus shows a zone within which the greater part of the combined neuraminic acid is precipitated. This coincided with the precipitation of the greater part of the material most active in restoring tissue excitability. In Fig. 1 the two properties are compared in eight fractions. The correlation is evident; it is also apparent that fraction V forms an exception in having an ability to restore tissue excitability which is disproportionately large for its neuraminic acid content. Here the properties of serum albumin are relevant. A crystalline specimen of the albumin was found (Marks & McIlwain, 1959) to have a limited ability to restore excitability to tissues kept in media at 0°. In quantities between 5 and 60 mg./ml. of medium the specimen was shown to increase respiratory response by 10–17%, and no more; though 5 and 10 mg./ml. gave values of 11 and 12%, 60 mg./ml. gave only 10%. The albumin specimen has now been found to be almost completely free of neuraminic acid derivatives (Table 1). Albumin constitutes the greater part of fraction V (Cohn *et al.* 1946) and it therefore appears reasonable, in appraising the contribution of neuraminic acid derivatives to the properties of fraction V, to allow for the properties of the albumin. This has been done in the two open circles of Fig. 1, when the correlation between restoring ability and neuraminic acid content becomes closer.

Ganglioside preparations and a sialomucopolysaccharide

The major neuraminic acid-containing substances of the brain are the gangliosides; their constituents are not yet completely defined (see Experimental section) but include also galactosamine, glucose, galactose and ceramides (sphingosine-fatty acid amides). Three ganglioside preparations, made by different methods and described above, have been examined for their ability to restore excitability to cerebral tissues kept at 0°. All were active (Table 2). Maximal restoration was obtained with 0·15 mg. (/ml.) of a preparation which corresponded to an addition of 36 μg. (/ml.) of combined *N*-acetylneuraminic acid. Comparison with the ability of the serum fractions to restore excitability (Fig. 1) shows that when the activities of the two categories of material are expressed in terms of their neuraminic acid content, the ganglioside preparations are markedly more potent. *N*-Acetylneuraminic acid itself (0·5 mg./ml.) had little or no ability to restore excitability.

A specimen of a sialomucopolysaccharide, kindly given by Professor W. T. J. Morgan, has, however, shown activity. The compound (Pusztai & Morgan, 1960) was isolated from ovarian-cyst fluid, and contains, in addition to a sialic acid, hexosamine, reducing sugars and fucose. At a concentration of 1 mg./ml. it showed distinct but limited ability to restore excitability to the tissue kept at 0°. Its activity (Table 2) was similar, in terms of neuraminic acid content, to that of the blood-plasma fractions.

Inhibition by basic proteins and a polypeptide; restoration and antagonism by the gangliosides

Histone and protamine preparations at 2–10 μM were previously shown to inhibit metabolic responses by cerebral tissue to applied electrical pulses; excitability was restored to the tissue by further addition of plasma fraction IV-4 (McIlwain, 1959*a*). Results are now reported (Fig. 2) with a laboratory preparation of calf-thymus histone, for which I am indebted to Dr L. S. Wolfe, and with a synthetic polypeptide. The freshly prepared histone was inhibitory in concentrations similar to those at which the previous specimen acted; neither of these

Table 2. *Restoration of response by materials added to tissue kept at* 0°

After exposure at 0° (see Experimental section), slices (45 mg.) were placed in electrodes in manometric vessels containing the added substances in 3·5 ml. of medium. Respiratory rate was measured for 40–45 min., after which pulses were applied for 40–45 min. and the measurement was continued.

Substance added	Weight added (mg./ml. of medium)	Respiratory rate (μmoles of O_2/g./hr.) Without pulses	With pulses	Increase in respiratory rate with pulses (%)
None	—	47±3 (6)	59±5 (4)	25±6 (4)
Plasma fraction IV-4	5	47 (3)	78 (3)	66
Ganglioside preparation I	0·4	49 (2)	86 (2)	75
	0·15	51±4 (4)	92±6 (4)	80±9 (4)
	0·06	48 (2)	73 (2)	52
Ganglioside preparation II	0·4	49 (2)	87 (2)	77
Ganglioside preparation III	0·8	47 (2)	89 (2)	89
Ganglioside preparation IV	0·4	48 (3)	90 (3)	88
Sialomucopolysaccharide	1·0	46±3 (4)	71±5 (4)	53±8 (4)
	0·3	46 (2)	65 (2)	41

preparations nor the protamine has been examined and purified by methods appropriate to large molecules, which have shown that histone preparations contain several distinct basic proteins (Luck, Rasmussen, Satake & Tsvetikov, 1958). The synthetic poly-L-lysine was, however, of similar activity to the protamine, and it would seem that the inhibition being investigated is a fairly general property of such basic polypeptides.

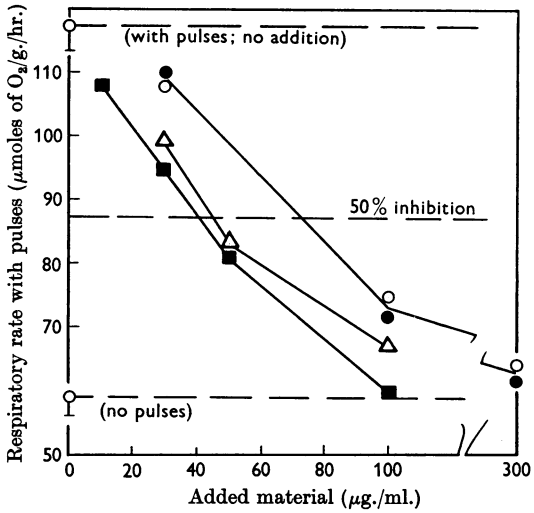

Fig. 2. Depression of respiratory response to pulses by the sulphates of calf-thymus histone (two specimens: ○ and ●), the protamines clupein and salmine (■) and by poly-L-lysine hydrochloride (△; mol.wt. approx. 5000). The substances were present in 3·5 ml. of media to which tissues (approx. 45 mg.) were added. Respiratory rate was measured first without pulses, and was little affected by the additions; this value is given by the lower broken line, with S.D. indicated on the ordinate. Pulses of 10 v peak potential and time constant 0·4 msec. were applied at 100/sec. during a second period, giving the rates recorded. The rate with pulses but without added substances is given by the upper broken line, again with S.D.

The gangliosides have been examined as agents which might restore excitability to cerebral tissues whose respiratory response was inhibited by protamine. Inhibition was first established by incubating tissue samples in media containing 150 μg. of protamine/ml., which was shown in other experiments to prevent completely the response to electrical pulses. The medium was then removed and the tissues, vessels and electrodes were washed with protamine-free media. In some vessels of an experiment, incubation was then continued in fresh, protamine-free solution; the tissue in these vessels remained inexcitable (Table 3). To other vessels ganglioside preparations were added, and in these response occurred. A concentration of 0·14 mg./ml. gave nearly maximal restoration of response.

The gangliosides have been investigated also as agents which might prevent the action of the basic proteins (Table 4). In these experiments, solutions of gangliosides and protamine were made in the usual metabolic media, and normal cerebral tissues subsequently placed in the solutions. Ganglioside preparations were then capable of preventing the inhibition usually caused by the protamine. The quantities of material involved were similar to those of the experiments of the preceding paragraph. In addition, the basic proteins inhibit the normal action of 50 mM-potassium salts on cerebral tissues (McIlwain, 1959*a*); a ganglioside preparation prevented this action also of the protamine (Table 4).

Complex-formation between basic proteins and neuraminic acid-containing compounds of large molecular weight

The fractions of blood-plasma proteins that restored excitability to cerebral tissues kept in media at 0° formed precipitates with histone and prot-

Table 3. *Ganglioside preparations restoring excitability to protamine-treated tissues*

Slices of guinea-pig cerebral cortex were incubated in electrodes in media with or without added protamine sulphate (0·15 mg./ml. when added; see Experimental section) for 15 min., after which these media were removed and replaced by others without protamine but containing gangliosides when indicated. Respiratory rates were measured during two successive periods of 45 min., during the second, pulses of the characteristics quoted in Fig. 2 were applied. Values are the averages of two or three observations which agreed to within 5%, except when followed by S.D. and number of observations.

Additional substances present		Respiratory rate during successive periods (μmoles of O_2/g./hr.)	
During preincubation	During respiratory measurements (mg./ml.)	Without pulses	With pulses
None	None	57±3 (5)	108±6 (5)
None	Gangliosides II, 0·43	59	104
Protamine	None	56±2 (4)	58±5 (4)
Protamine	Gangliosides II, 0·43	60	91
Protamine	Gangliosides I, 0·57	54	97
Protamine	Gangliosides I, 0·14	56	88

amine preparations (McIlwain, 1959*a*). The sialomucopolysaccharide also has now been found to exhibit this property in high dilution (Table 5). Visible precipitation was given in neutral solutions at 60 μg./ml., with the protamine or histone at 100 or 300 μg./ml. These are the concentrations of the basic proteins about minimal for maximum inhibition under the conditions of the preceding experiments. Precipitation increased with increase in concentration of the sialomucopolysaccharide to about 200 μg./ml., but then diminished, and at the higher concentration of Table 5 precipitation did not occur.

In similar experiments ganglioside preparations yielded either no precipitate or only slight precipitates, much less than the maximum obtained with the sialomucopolysaccharide, though concentrations of gangliosides between 10 and 1000 μg./ml. were examined. Two types of ganglioside preparations were used (see Experimental section), one made by a procedure yielding peptide-containing preparations and the other peptide-free; they behaved similarly towards the proteins. Interaction between the gangliosides and the proteins was, however, shown by the addition of further reagents. Picric acid, known to form insoluble salts with protamines and histones (Cohnheim, 1900), and used in the previous studies (McIlwain, 1959*a*), gave an evident precipitate with the proteins (50 μg./ml.) in the buffered media of the present experiments; fluorescein gave a bulkier and darker precipitate (see Experimental section). When, however, small quantities of the gangliosides were present with the protamine or histone, precipitation with picric acid or fluorescein was prevented. The concentrations involved are shown in Table 5.

Moreover, when the sialomucopolysaccharide was added to either of the basic proteins in a quantity sufficient first to form a precipitate and then to

Table 4. *Ganglioside preparations preventing inhibition by protamine*

Slices of guinea-pig cerebral cortex were placed in glycylglycine media in manometric vessels. The media already contained the additional substances of the Table (weights of protamine refer to the sulphate) before the tissues were added. In Expts. A, the tissues were in electrodes; in Expts. B they were floating freely. After 40–50 min. incubation, during which respiration was measured, electrical pulses (as in Fig. 2) were applied to the tissues in electrodes, and additional M-KCl (making the media 50 mM in K^+) tipped from side arms to the other tissues; respiratory measurements were continued for a further 40–50 min. Results are quoted as mean values from the number of observations in parentheses (with standard deviation) or from two to three observations.

Group of expts.	Additional substances present (mg./ml.)	Respiratory rate (μmoles of O_2/g./hr.) 1st period	2nd period
A, B	None	64±3 (6)	131±6 (6)
A	Protamine, 0·11	63±3 (4)	79±5 (4)
A	Protamine, 0·11; ganglioside I, 0·43	63±3 (4)	131±4 (4)
A	Protamine, 0·11; ganglioside I, 0·14	65	108
A	Protamine, 0·11; ganglioside II, 0·43	62	130
B	Protamine, 0·05	65	89
B	Protamine, 0·10	64	69
B	Protamine, 0·10; ganglioside I, 0·29	63	115

Table 5. *Precipitation reactions of basic proteins with a mucopolysaccharide and with ganglioside preparations*

To a series of tubes containing the protein sulphates dissolved in 33 mM-glycylglycine or in tris-buffered media were added varied quantities of the other components in the same media. About 1 hr. after making these mixtures the fluorescein or picric acid solutions were added (see Experimental section). This caused marked precipitation with the basic proteins alone; the other components either (*a*) lessened the extent of these precipitates or (*b*) completely or nearly completely prevented them.

Protein sulphate (μg./ml.)	Other component	Concentration (μg./ml.) of other component causing just noticeable ppt.	maximal ppt.	redissolving of ppt.	with fluorescein or picric acid: (*a*) decreased ppt.	(*b*) little or no ppt.
Protamine, 120	Sialomucopolysaccharide	60	230	720	230	720
Protamine, 120	Ganglioside IV	Little or no precipitate			55	300
Histone, 300	Sialomucopolysaccharide	60	170	350	120	350
Histone, 300	Ganglioside IV	Little or no precipitate			95	300

begin to dissolve it, the basic protein was again protected from precipitation by picric acid or fluorescein. It is therefore suggested that the ganglioside preparations form soluble and fairly firmly bound complexes with the protamines and histones. These interactions are exhibited under the conditions in which their effects on tissue excitability are shown, and in concentrations of 50–300 μg./ml.

By using the precipitation reactions in the same semi-quantitative way, combination between the basic proteins and a constituent of cerebral tissue has been shown to occur during metabolic experiments. After incubating slices of approx. 50 mg. under the conditions of Tables 3 or 4 with protamine (75 or 100 μg./ml.), the slices were removed and fluorescein was added to portions of the medium. The reaction given was markedly diminished by the tissue, and after incubation corresponded to only about one-third of its initial value. When protamine (150 μg./ml.) was added, the residual reaction corresponded to about half the protamine added. This suggests an interaction with about 4 μg. of protamine for each milligram of tissue. All these concentrations of protamine gave complete or almost complete inhibition of response. Because of the finding, which has been previously described, that gangliosides in solution can prevent reaction between protamine and fluorescein, these observations do not decide whether combination occurs at the tissue or in solution, and experiments are in progress to determine this.

DISCUSSION

Keeping cerebral tissues in cold media was chosen for study as being one of the mildest of procedures causing loss of excitability. It does, however, diminish the contribution of metabolically derived energy to the maintenance of structure and of concentration gradients, and possibly in this way permits the interaction of cell constituents which themselves possess mutual affinity. The gangliosides and histones have been shown to have great mutual affinity. They are large molecules, the gangliosides with some 160 acidic groups per molecule and the basic proteins with 20–30 basic groups per molecule; and, as indicated in Table 5, they form complexes in high dilution in the neutral aqueous solutions in which they affect tissue excitability. The simplest interpretation of the properties of these two components, as exhibited after isolation and separate addition to cerebral tissues, is to suppose that the histones act as inhibitors through their combination with tissue gangliosides, and consequently that the gangliosides are normally involved in tissue excitability.

A wider survey of tissue anions is, however, needed before this interpretation can be regarded as established. In addition to gangliosides, the brain contains other neuraminic acid derivatives (Svennerholm, 1956; Brante, 1959). It also contains other acidic substances of large molecular weight. A recent appraisal of ion balance in the brain as a whole (McIlwain, 1959*b*) indicated that, even including recently recognized constituents such as *N*-acetylaspartic acid, some 80 μequiv. of anions/g. of tissue remained to be accounted for by materials such as lipids or proteins. Much of this material, to judge by the properties of cerebral extracts as agents restoring tissue excitability, is not involved in the present phenomena, but certain constituents including the sulphatides are to be noted. The sulphatides (see McIlwain, 1959*b*) are, however, present at greater concentration in white matter than in grey, and a substance concerned with tissue excitability in the present sense would be expected to be distributed in favour of grey matter. The gangliosides are so distributed and in addition occur almost exclusively in neural rather than in the other animal tissues examined (Long & Staples, 1959).

Interacting quantities of gangliosides and basic proteins

Data on the substances examined in the present study are assembled in Table 6. The specimens of gangliosides and basic proteins which have been used are naturally occurring mixtures rather than pure substances; this is also true for the specimens to which many of the data refer, and is to be borne in mind in the following appraisal.

(1) When protamine and poly-L-lysine inhibited the tissue's response by 50 %, they are seen to have been added in molar amounts which are 30–50 % of the molar quantity of *N*-acetylneuraminic acid present in the gangliosides of the tissue on which they acted. Moreover, this quantity of protamine (about 0·7 μmole/g. of tissue) was shown by the experiments above to combine with the tissue or with a tissue constituent, concomitantly with the inhibition of tissue excitability.

(2) When (see Table 5) gangliosides protected the protamine of the solutions of Table 5 from precipitation by fluorescein or picric acid, a quantity of ganglioside containing (see Table 6) about 2 equiv. of *N*-acetylneuraminic acid per mole of protamine partially prevented precipitation; about 8 equiv. were needed to prevent precipitation completely.

(3) Gangliosides, as agents restoring response, acted in quantities within the range to be anticipated from the values of (1) and (2): 50 % restoration was given by solutions containing about 2·5 times the quantity of gangliosides contained in the tissue under test.

(4) The poly-L-lysine of molecular weight similar to the protamine, but with more basic groups, was

Table 6. *Quantities of materials whose interaction conditions response to pulses*

Substance examined			Amount* of substance (*A*) involved in (*C*)		Interacting quantities derived from (*B*) and (*D*)	
(*A*)	(*B*) Mol.wt. or equiv.wt.	(*C*) Property of substance *A* which is measured	Concentration in solution (μg./ml.)	(*D*) Expressed in relation to tissue wt. (μg./g. fresh wt.)	(μmoles/g. of tissue)	(μequiv./g. of tissue)
Protamine sulphate	Mol.wt. 5000† Equiv.wt. 270 or 830†	50% inhibition of response	40	3100	0·62	11·5; 3·7
Poly-L-lysine hydrochloride	Mol.wt. 4950† Equiv.wt. 166‡	50% inhibition of response	50	3900	0·79	23·5
Histone sulphate	Mol.wt 1–2 × 10⁴§ Equiv.wt. 371**	50% inhibition of response	70	5450	0·3–0·5	14·7
N-Acetylneuraminic acid of gangliosides	Mol.wt. 309	Quantity in cerebral cortex‖	—	525	1·7	—
Gangliosides	Mol.wt. 2 × 10⁵¶ Equiv.wt. 1200¶	50% restoration of response	70	5450	0·027	4·5

* The amounts of substances are derived from the data of Tables 2–4 and Fig. 2.
† Callanan, Carroll & Mitchell, 1957; Carroll, Callanan & Saroff, 1959. Salmine has 18 to 19 basic groups, but 6 strongly basic sites.
‡ Wetlanjer & Stahmann, 1953; Katchalski & Sela, 1958; the equivalent weight is quoted on the assumption that all basic groups are effective.
§ Crampton, Stein & Moore, 1957.
‖ Long & Staples, 1959.
¶ Folch, Arsove & Meath, 1951; Rosenberg & Chargaff, 1958.
** See Experimental section.

active in approximately the same quantity by weight. A greater weight but rather smaller molar quantity of histone was effective.

Relationship of the basic proteins to the tissue

To understand these interactions in detail is likely to require more specific knowledge of the ionization and affinities of the soluble reactants, and also of chemical and geometrical factors concerned in localization of the relevant acidic groups in the tissue. As well as the results of the accompanying paper (Wolfe & McIlwain, 1961), two groups of observations with erythrocytes appear apposite. The main acidic groups which condition the surface charge of these cells are likely to be neuraminic acid derivatives, since they are removed by neuraminidases (Pye, 1955; Gottschalk, 1958; Klenk, 1958). Combination of lysine polymers (including that used in the present study) with erythrocytes has been examined by Katchalsky, Danon, Nevo & de Vries (1959), whose evidence suggested that the molecules of the lysine polymers are fully extended in aqueous solutions, through mutual repulsion of the many positive charges of the molecules. The polymers appeared to become attached to acidic groups of the cell surface by basic groups near one end of the polymer, and to act as agglutinating agents through attachment at the two ends to the surface of two erythrocytes, which were then observed electron-microscopically to be separated by bridges of material about the length of the molecules of the lysine polymers used. In relation to the acidic groups of the cells, the polylysine is presumably then acting as a monobasic or dibasic substance in spite of its many positive charges, a situation implied by some of the interacting ratios observed in cerebral tissues and discussed above.

Evidence has been given by van Heyningen (1959) suggesting that gangliosides may constitute the site of attachment of tetanus toxin to neural tissues. van Heyningen (1959) emphasizes the specificity of the interaction of the gangliosides with the toxin rather than with other proteins. The toxin is indeed effective in minute amounts. However, it is feasible that a firmness of attachment exhibited by the toxin (which is not strongly basic) on account of specific structural relationships to the gangliosides, may be paralleled by that conferred by the multiple positive and negative charges which are the probable basis of the present interactions. These are not dependent on details of protein structure, for a protamine, a histone and an amino acid polymer are all capable of acting as the basic component. Whether the gangliosides may exhibit a special relationship to the cerebral histones is part of an investigation of specificity now in progress. That the cerebral histones, after migration from the nucleus, can condition tissue excitability may be

adventitious but may carry greater significance. It is known that nuclear size and composition in neurons undergo changes according to the level of neural activity; in other types of cell the nuclear proteins undergo changes during cell division. The possible modes by which histones, in their new site, may condition excitability will be realized from the preceding discussion to be manifold; inhibition by protamine is affected by calcium-ion concentration and is accompanied by change in tissue fluids (McIlwain, 1960*b* and unpublished work).

SUMMARY

1. Cerebral tissues kept in cold media do not give their normal respiratory response to pulses when subsequently brought to 37°. Response was restored by including certain fractions of blood-plasma proteins in the media during incubation. The potency of different plasma fractions in restoring response was correlated with their content of neuraminic acid derivatives.

2. Other substances of large molecular weight and containing neuraminic acid also restored the tissue's response: a sialomucopolysaccharide and preparations of gangliosides from the brain which were active at a concentration of 150 μg./ml. of medium.

3. An analogous unresponsive state was induced in normal cerebral tissues by incubation with protamines, histones or a synthetic poly-L-lysine at a concentration of 30–150 μg./ml. Response here also, after inhibition by protamine, was restored by gangliosides. Prior mixture of an amount of protamine normally inhibitory, with a little more than its own weight of gangliosides, prevented the protamine from exerting its inhibition.

4. Concentrations of gangliosides which restored response to the tissue kept cold or with protamine, when added to protamine without tissue, prevented the protamine being precipitated by picric acid or fluorescein. This was attributed to complex formation between gangliosides and protamine; similar phenomena were shown with inhibitory concentrations of histone, and also between the sialomucopolysaccharide and the basic proteins.

5. The quantities of the basic proteins and gangliosides which interact in conditioning the response of a tissue specimen are compatible with the proteins inhibiting by combination with the gangliosides of the specimen. The possibility is discussed of such a mechanism operating when response is lost on keeping the tissue in cold media or with protamine.

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