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An Attachment of Protamines to Cerebral Tissues, Studied in Relation to Gangliosides, Suramin and Tissue Excitability

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Cerebral tissues incubated in oxygenated glucose media normally respond to electrical pulses by a number of metabolic changes (McIlwain, 1951, 1959*a*); if basic proteins are added to such incubation media, response to pulses is diminished or lost (McIlwain, 1959*b*). Loss of response is accompanied by a change in the tissue gangliosides, of which a greater proportion becomes fixed to the tissue in a form not immediately extractable by chloroform–methanol mixtures (S. Balakrishnan & H. McIlwain, in preparation). Concomitant changes in the fluids of the tissue have also been observed, as is reported below. Small quantities of basic proteins suffice to cause the loss of excitability, for example 0·15 mg. of a protamine sulphate/ml. of incubation medium. In these concentrations protamine can, however, be detected by precipitation reactions before the medium is incubated with cerebral tissues. After incubation, the protamine so detectable in the medium diminished (McIlwain, 1960, 1961).

The simplest interpretation of these phenomena is that the protamines are taken up from the medium by combination with the tissue gangliosides, into complexes not extractable with chloroform–methanol mixtures. Other explanations are not, however, excluded, for gangliosides form soluble complexes with the basic proteins (McIlwain, 1960, 1961). Direct evidence for attachment of basic proteins to tissue slices has therefore been sought by analysis of the incubation medium and of the tissue itself. Preliminary experiments showed that it was possible to determine protamines in such materials by their constituent arginine, as was done by Wolfe & McIlwain (1961), and this has been made the basis of the present study.

Excitability can be restored to tissues which have been exposed to protamines, by subsequent incubation with ganglioside-containing solutions; also, if added together with adequate quantities of gangliosides, protamines are no longer inhibitory. Distribution of protamines between tissue and solution under these conditions also has been examined. Certain other substances act as do gangliosides in restoring tissue excitability, though greater concentrations are required of the other antagonistic agents (McIlwain, 1960, 1961 and unpublished work). One of these substances, the sodium salt of bis-(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamine-4:6:8-trisulphonate) carbamide, has been included in the present study. It is the antitrypanosomal drug suramin.

EXPERIMENTAL

Tissue metabolism. Guinea-pig brains were obtained and slices of cortex prepared from the cerebral hemispheres, and weighed, as described by Marks & McIlwain (1959). Slices from one animal were used in each of the experiments quoted in the Results section. Tissue weighing 50–70 mg. was incubated at 37·5° in 3·5 ml. of the phosphate medium of Rodnight & McIlwain (1954), oxygenated and with the additions noted in individual experiments. Respiration was followed manometrically in conventional Warburg apparatus, readings being taken at 5 min. intervals. Immediately after incubation, the contents of the main compartment of a vessel were tipped into a shallow dish and the tissue was transferred with a spatula to a second dish of medium and immediately to a tube for hydrolysis. This was done successively and promptly with each vessel. Electrical stimulation employed the silver-grid electrodes of Ayres & McIlwain (1953), to which were applied alternating condenser pulses of 10 v peak potential, 0·4 msec. time constant, at 100/sec.

Arginine assay. Tissues, and fluids after centrifuging for 5 min. at 800*g*, were hydrolysed according to Wolfe & McIlwain (1961) to liberate protein arginine. Incubation fluids (1 ml.) were placed with 1 ml. of 10N-HCl and 30 μl. of 12·5% (w/v) titanous chloride in 16 mm. × 150 mm. Pyrex tubes. The tubes were sealed with a blowlamp and kept at 110–120° for 4 hr. Tissues were ground in similar tubes with 1 ml. of 10N-HCl with a glass pestle, which was subsequently washed in with 0·5 ml. of 10N-HCl and 1·5 ml. of water, 40 μl. of the titanous chloride was added and hydrolysis completed as described above.

After hydrolysis, the contents of the tubes were neutralized (shown by maximal precipitation of titanium hydroxides) with 6N-NaOH, filtered and diluted for assay. Diluted extracts were found to be stable for 24 hr. at 0°, but for longer storage it was found best to keep the specimens in their sealed tubes before hydrolysis and at 0°. Arginine was determined by Satake & Luck's (1958) method, with internal standards in the range 20–60 μM-arginine. Estimations were carried out in triplicate and a reagent blank was included with each group. Because of

the removal of a small precipitate by centrifuging, not all arginine of the incubation mixtures is accounted for, e.g. in the experiments of Table 1. The centrifuging was carried out in order that tissue arginine could be compared with arginine in solution in the ambient fluid, and not in suspension.

Determination of suramin. In fluids, suramin was determined according to Gage, Rose & Scott (1948), with a Unicam spectrophotometer and measuring E at 505 mμ. Tissues (approx. 60 mg.) were ground in 3 ml. of 0·1N-NaOH in a test-tube homogenizer and after 15 min. at 0° the mixture was centrifuged (15 min., 800g). To 2 ml. of supernatant were added: 0·5 ml. of 0·1M-KH_2PO_4 brought to pH 7·4 by NaOH; 20μl. of Universal indicator (British Drug Houses Ltd.); 0·1N-HCl to neutrality. Of the neutralized extract, 0·2 ml. was taken for analysis as described above. The tissue extracts gave an apparent suramin content of 1·0–1·5 mg./g. of tissue when no suramin had been added; this blank was not subtracted from the values quoted. Recovery of suramin added to the alkaline tissue extracts was 92–95%.

Materials. L-Arginine monohydrochloride was from L. Light and Co. Ltd. Two specimens of protamine sulphate were used: the analysed specimen of the clupein salt, from herring, described by McIlwain (1960, 1961) and found by the above-described assay to yield 0·41 g. of arginine/g.; salmine sulphate (British Drug Houses Ltd.), which yielded 0·43 g. of arginine/g. Two ganglioside preparations were made from ox cerebral cortex largely by Folch, Lees & Sloane-Stanley's (1957) method, as described by McIlwain (1960, 1961). One specimen contained 26·5% of N-acetylneuraminic acid when assayed according to Long & Staples (1959; for additional details see McIlwain, 1960, 1961); a second, for which we are indebted to Dr S. Balakrishnan, contained 28%; these materials are mixtures of more than one molecular species (Meltzer, 1958; Folch & Lees, 1959; Klenk & Gielen, 1960; D. A. Booth, personal communication). Suramin was obtained from Imperial Chemical Industries Ltd. (Pharmaceuticals Division) and from Farbenfabriken Bayer A.-G. We are indebted to Dr F. L. Rose of Imperial Chemical Industries Ltd. (Pharmaceuticals Division) for 2-p-dimethylaminostyryl-6-acetamidoquinoline methochloride (I.C.I. 5302; SQ 24 of Browning, Cohen, Ellingworth & Gulbranson, 1926) and for indicating its use in determining suramin. Other materials obtained were of A.R. grade.

RESULTS

Arginine content of cerebral tissues

Slices of guinea-pig cerebral cortex, cut and floated in phosphate medium and drained and weighed but not incubated, contained 7·8 ± 0·73 mg. of arginine/g. of tissue from one animal, and from another, 7·7 ± 0·54 (S.D.; six and five slices respectively). After incubation for 60 min. at 37·5° in oxygenated phosphate medium, 6·16 ± 1·35 mg. of arginine/g. of tissue was found in 15 tissues from eight guinea pigs; the incubation fluids then contained arginine equivalent to 1·19 ± 0·47 (15) mg./g. of tissue.

These values indicated the feasibility of following the fate of added protamine by arginine assays, for protamine sulphate diminishes tissue excitability when added at 0·05–0·25 mg./ml. of incubation fluid, with a tissue/fluid ratio of 20 mg./ml. (McIlwain, 1960, 1961). This involves addition of 2·5–12·5 mg. of protamine sulphate or 1·1–5·5 mg. of arginine/g. of tissue. Protein constitutes about 10% of the brain, and mixed cerebral proteins contain 5–5·4% of arginine (Block, Jervis, Bolling & Webb, 1940), indicating the greater part of the arginine determined above to be derived from protein; the free arginine is small (Blass, 1960).

Table 1. *Protamine uptake by guinea-pig cerebral cortex*

Protamine was added to the phosphate medium (3·5 ml.) before the tissue was placed in it. Respiration was measured at 37·5° for 60 min. (giving rates of 58–65 μmoles of O_2/g. of tissue/hr.), after which arginine was determined. For other details, see the Experimental section.

		Tissue		Fluid	
Expt. no.	Substance added (mg./ml.)	Initial wt. (mg.)	Final arginine (mg./g.)	Arginine (μg./vessel)	Arginine (mg./g. of tissue)
1	None	50	6·2	53	1·1
		70	5·4	99	1·4
	Clupein SO_4 (0·06)	60	10·3	143	2·4
		70	7·2	236	3·4
	Clupein SO_4 (0·1)	65	9·6	124	1·9
		60	12·5	178	3·0
2	None	—	5·9 ± 0·8 (4)	—	0·9
	Clupein SO_4 (0·1)	63	7·7	89	1·4
		54	7·5	320	5·9
3	None	63	6·6	44	0·7
	Salmine SO_4 (0·1)	56	8·8	107	1·9
		43	8·4	125	2·9
	Salmine SO_4 (0·2)	59	10·5	258	4·4
		49	10·4	254	5·2

Table 2. *Course of protamine uptake*

Tissues were incubated for 10 min. in protamine-free media; clupein sulphate (0·214 mg./ml.) was then added and, after the intervals indicated, tissues were removed for analysis. Each result is the mean of duplicate determinations on each of two slices; other details are as given for Table 1.

Expt. no.	Arginine of the protamine SO_4 added (µg./ml. of medium)	Time of incubation with protamine (min.)	Arginine content: Tissue (mg./g.)	Arginine content: Fluid (mg./g. of tissue present)
1	0	10	5·0	—
	110	10	5·7	—
	0	30	5·0	—
	110	30	6·7	—
2	0	30	3·8	1·4
	108	30	7·7	8·5
	0	60	3·9	2·6
	108	60	7·8	8·1

Table 3. *Protamine uptake in the presence of gangliosides*

Tissues were incubated for 1 hr. in 3·5 ml. of phosphate medium containing from the start the protamine with or without gangliosides. Each value for tissue content is the mean of four determinations: two estimations on each of duplicate slices. Each value for fluid arginine is the mean of two estimations. Other details are as given for Table 1.

Expt. no.	Substances added (mg./3·5 ml.)	Arginine content: Tissue (mg./g.)	Arginine content: Fluid (mg./g. of tissue)
1	None	8·2	1·8
	Clupein SO_4 (0·75)	10·4	7·2
	Clupein SO_4 (0·75); ganglioside (2)	6·7	8·8
2	None	5·8	0·6
	Clupein SO_4 (0·75)	9·8	5·4
	Clupein SO_4 (0·75); ganglioside (1)	7·6	6·1
3	None	6·3	—
	Ganglioside (1)	5·4	—
	Ganglioside (2)	4·5	—

Table 4. *Gangliosides added to protamine-treated slices*

The design of Expts. 1 and 2 was that of Table 3 of McIlwain (1961). In the first 15–20 min. period, respiratory rates were 58–65 µmoles of O_2/g. of tissue/hr.; the media were then replaced by those of the second period (40 min.) and rates were 49–60 µmoles of O_2/g./hr. In Expt. 3, after 30 min. incubation, constituting the first period, ganglioside was added from the side arms of the vessels indicated and an equivalent volume of medium was added to the others; incubation was continued for a further 30 min. Each result is a mean of four determinations: two estimations on each of two slices.

Expt. no.	Additional substances present (mg./3·5 ml.): First period	Additional substances present (mg./3·5 ml.): Second period	Arginine of tissue (mg./g.)
1	None	None	4·5
	Clupein SO_4 (0·75)	None	6·4
	Clupein SO_4 (0·75)	Ganglioside (2)	6·7
2	None	None	4·2
	Clupein SO_4 (0·75)	None	5·5
	Clupein SO_4 (0·75)	Ganglioside (2)	5·3
3	None	None	5·5
	Clupein SO_4 (0·75)	Clupein SO_4 (0·75)	7·8
	Clupein SO_4 (0·75)	Clupein SO_4 (0·75); ganglioside (2)	9·1

Incubation with protamine; effect of gangliosides

Slices of cerebral cortex incubated in media containing 0·06–0·2 mg. of protamine sulphate/ml. increased in arginine content (Table 1). The increase took place with both protamine specimens examined; uptake was marked between 10 and 30 min. of incubation but had increased little at 60 min., although appreciable protamine remained in solution (Table 2).

The concentrations of gangliosides found (McIlwain, 1960, 1961) to interact with the concentrations of protamine now being studied were of 0·1–0·5 mg./ml. The effect of such additions has been investigated in three ways. In the first (Table 3) gangliosides were mixed with protamine before the tissues were added for incubation. After incubation in such mixtures, the usual uptake of protamine was found to have been partly or completely blocked, according to the amount of ganglioside added. Possibly the higher concentration of ganglioside withdrew arginine-containing material from the tissue itself. This was the case when tissues were incubated with gangliosides only. In the second type of experiment (Table 4, Expts. 1 and 2) tissues were initially incubated in protamine-containing solutions for 15–20 min. The media were then removed and replaced either by media without addition or by media with gangliosides. In this situation, gangliosides can restore tissue excitability (McIlwain, 1960, 1961). They did not, however, diminish the tissue content of protamine. In a further experiment (Expt. 3, Table 4) gangliosides were added during incubation and without prior removal of the protamine-containing solution. The results suggested that addition of the gangliosides promoted a further binding of protamine to the tissue.

Protamine and suramin

Suramin at 0·25–2 mg./ml. of incubation fluid partly restores excitability to tissues previously exposed to protamine, and its effect on tissue protamine has accordingly been examined (Table 5). Analyses for tissue arginine carried out after incubation in protamine–suramin mixtures (Expt. 1) showed that suramin could diminish the uptake of protamine; it also formed a precipitate with protamine. However, protamine, once attached to the tissue, was not removed by subsequent incubation with suramin (Expts. 2–4, Table 5). The findings with suramin thus paralleled those with gangliosides.

The suramin content of tissues treated in this fashion has also been estimated. Much suramin was found to be taken up by the tissue, whether or not it had first been incubated with protamine (Table 6). Although the quantity of suramin and the period of incubation with it were varied in the different experiments, no evidence was found for protamine causing a major increase in the uptake of suramin by the tissue. The only indication of such uptake is in Expt. 3, Table 6, and is not large.

Other observations

In Tables 1–6 tissue constituents have been expressed in terms of the weight of the slices determined at the commencement of the experiments immediately before incubation. Such slices normally increase in weight during incubation by

Table 5. *Suramin and tissue protamine*

Expt. 1 was performed as described in Table 3, ganglio side being replaced by suramin. Expts. 2–4 were performed as described in Table 4, Expts. 1 and 2, ganglioside being replaced by suramin.

	Substances added (mg./3·5 ml.)		
Expt. no.	First period	Second period (suramin)	Arginine content (mg./g. of tissue)
1	None	—	5·0
	Clupein SO_4 (0·75)	—	7·0
	Clupein SO_4 (0·75); suramin (0·75)	—	5·3
2	None	0	5·1
	Clupein (0·75)	0	7·7
	Clupein (0·75)	0·75	6·8
3	None	0	4·7
	Clupein (0·75)	0	6·0
	Clupein (0·75)	3·5	6·1
4	None	0	4·3
	Clupein (0·75)	0	5·2
	Clupein (0·75)	7·0	5·6

Table 6. *Attachment of suramin to cerebral tissue*

Expts. 1 and 2 were carried out as described in Table 4, Expts. 1 and 2. In Expt. 3, suramin was placed in the side arms of the Warburg vessels and tipped in after preincubation for 20 min. After incubating for a further 5 min., the vessels were removed and suramin was estimated as described in the Experimental section. The tissues give an apparent suramin value when none is added.

	Substance added (mg./3·5 ml.)		Suramin or apparent suramin	
Expt. no.	First period (clupein SO_4)	Second period (suramin)	Tissue (mg./g.)	Fluid (mg./3·5 ml.)
1	0	0	0·7	0·014
	0	3·5	4·1	3·5
	0·75	3·5	3·9	3·4
2	0	0	1·5	0·02
	0	0·75	3·6	0·70
	0·75	0·75	3·3	0·66
3	0	0	1·48	0·016
	0	0·75	1·53	0·73
	0·75	0·75	1·86	0·56

absorption of fluid (Pappius & Elliott, 1956; Leaf, 1956; Cummins & McIlwain, 1961). Protamine in the concentrations employed in the present experiments has now been observed to diminish such uptake of fluid (Fig. 1). It also causes a small change in the appearance of the tissue, which at the end of incubation in protamine-containing solutions is slightly more whitish and opaque when compared with slices incubated without protamine; these retain to a greater extent their original pale, partly translucent, grey appearance. The change with protamine is seen best when slices with and without additions are floated together in a shallow, black-glazed dish; it becomes more obvious when larger quantities of protamine are added. Moreover, the changes both in appearance and in uptake of fluid are in part antagonized by added gangliosides (Fig. 1).

DISCUSSION

The action of protamine on the excitability of cerebral tissues (McIlwain, 1959*b*, 1960, 1961) has now been shown to be accompanied by the attachment of protamine to the tissue. Results of Table 1 indicate the tissue to acquire rather more than half the quantity of protamine added. As in these experiments tissues of 60 mg. were incubated in 3·5 ml. of fluid, a 60-fold concentration in the tissue is implied. This computation is approximate only, it being supposed that the protamine does not affect the distribution of other arginine-containing substances.

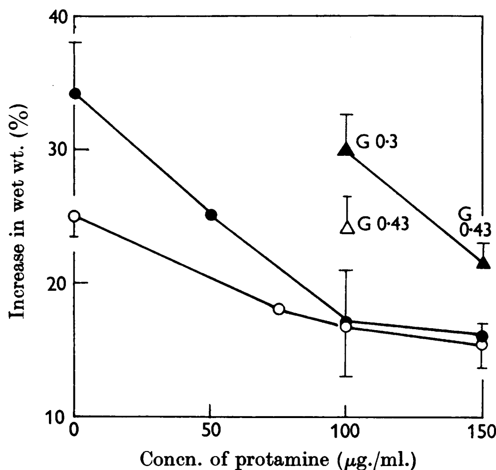

Fig. 1. Change in weight of guinea-pig cerebral cortex after incubation for 80 min. Clupein sulphate and gangliosides (G, mg./ml.) were present in the quantities indicated, in glycylglycine–glucose medium (McIlwain, 1959*b*), throughout the experiments. Electrical pulses, when indicated, were applied for the last 35–40 min. of the experiment. Tissue with protamine only: no pulses, ○; with pulses, ●. Tissue with protamine and gangliosides: no pulses, △; with pulses, ▲. Vertical lines extend from points to distances equivalent to the S.D. (four to six values); other points are means of two values.

A migration of the basic proteins native to cerebral tissues, which also leads to loss of tissue excitability, was previously concluded (McIlwain, 1959*b*; Wolfe & McIlwain, 1961; Wolfe, 1961) to involve attachment of the proteins to membrane structures. Evidence for this was given by analysis of fractions of ground cerebral tissues after differential centrifuging. As it is known that excitation in isolated mammalian cerebral tissues, as in other systems, is associated with ion movements (Cummins & McIlwain, 1961) across membranes which are normally polarized (Li & McIlwain 1957), attachment of the inhibitory proteins at the 'active' cell membrane involved in ion movement offers a reasonably direct picture of the actions of the proteins on tissue excitability. Basic polypeptides have been concluded to agglutinate erythrocyte suspensions by drawing together adjacent cells through attachment of the peptide molecules between the membranes of pairs of cells (Katchalsky, Danon, Nevo & de Vries, 1959). It appears feasible that such action on the part of protamine could account for the changes in appearance and in water content that it causes in the tissue. Surface membranes of cell processes in the rat cerebral cortex have been observed to be some 200–400 Å apart, and tubules of the endoplasmic reticulum to be some 200 Å in diameter (Gray, 1958, 1959).

The site of attachment of protamine is to be characterized chemically as well as in terms of cell structure; the previous suggestion that attachment was to ganglioside-containing materials (McIlwain, 1960, 1961) was based on extraction and fractionation of naturally occurring materials in search of compounds able to antagonize the inhibitory effects of the basic proteins. Antagonism by gangliosides is now seen to extend to other aspects of the interaction between protamine and tissue: to the attachment of protamine to tissue slices (Table 3) and to the effect of protamine on tissue fluids (Fig. 1). Certain related findings were, however, unexpected: when gangliosides were added to cerebral tissues which had lost excitability through incubation with protamine, excitability was largely restored (McIlwain, 1960, 1961) but protamine remained attached to the tissue (Table 3, this paper). There are even suggestions that gangliosides further increase the binding of protamine under these conditions.

Tissue gangliosides are being made the subject of an independent study (S. Balakrishnan & H. McIlwain, in preparation); the results of the parallel experiments with suramin may be dis-

cussed here. Suramin readily combines with many tissues and proteins of the body (Boursnell & Wormall, 1939; Spinks, 1948). Under conditions in which it increases the excitability of protamine-treated tissue, much suramin is taken up by the slices (Table 6). As both protamine and suramin are highly water-soluble substances, their attachment to the tissue is a very real phenomenon. The values of Table 6 imply a four- to ten-fold concentration of suramin in favour of the tissue. The attachment of suramin is largely independent of added protamine, but may be compared in quantity with the protamine which becomes attached under similar conditions. From Tables 3–5 incubation with 0·75 mg. of protamine/vessel is seen to lead to uptake of approx. 2·5 mg. of arginine/g. of tissue, or 6·1 mg. of protamine/g. This is approx. 1·2 μ-mole/g., and 7·2 or 22 μequiv./g., according to the basicity considered relevant (for sources of data see McIlwain, 1960, 1961). On incubation with 0·75 and 3·5 mg. of suramin/vessel, tissues take up 2 and 3·3 mg. of suramin/g. (Table 6). With a mol.wt. of 1466 these quantities correspond to 1·4 and 2·3 μmoles/g., or 8·2 and 13·5 μequiv./g. Computed in these different ways therefore similar quantities of acidic and basic substances are being attached to the tissue during these experiments. Suramin appears unlikely to simulate gangliosides in fashions other than its being a multivalent anion of large molecular weight, and the present experiments therefore contribute to the picture (McIlwain 1960, 1961) of negatively charged surface structures being required for the ion movements associated with tissue excitability.

SUMMARY

1. Protamine added to media in which cerebral cortical tissue is incubated is taken up by the tissue, which loses its metabolic response to electrical pulses. Uptake is small in 10 min. but nearly maximal in 30 min.

2. Uptake of protamine is diminished by concentrations of gangliosides which diminish the action of protamine on tissue excitability. Protamine already attached to the tissue is not, however, removed by subsequent incubation in ganglioside-containing solutions.

3. Protamine diminishes the uptake of fluid that normally occurs on incubating cerebral tissues *in vitro*, both with and without application of electrical pulses. Gangliosides oppose protamine in this respect also.

4. Suramin, which also diminishes the action of protamine, again prevents the binding of protamine by cerebral tissues but does not remove protamine already attached.

5. Suramin added to incubating fluids itself becomes attached to cerebral tissues; the quantity bound is independent of previous exposure of the tissue to protamine, but is similar to that of the protamine whose action it opposes.

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