Basic Proteins and the Potassium Movements and Phosphates of Cerebral Tissues

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An inexcitable condition in cerebral tissues is associated with migration of histones from the cell nuclei to material which appears to be derived from cell membranes (McIlwain, 1959; Wolfe & McIlwain, 1960, 1961). In ground cerebral tissues added histones and other basic proteins or polypeptides became attached to the same subcellular fractions, which were found to be rich in gangliosides (Wolfe, 1961). Tissue excitability was restored by adding gangliosides and certain related substances (McIlwain, 1960*a*, 1961*a*). Because of these indications of the chemical nature of the target or receptor for the basic proteins, knowledge of any metabolic concomitants of their action would be of value in indicating the role played by the gangliosides and possibly in revealing part of the mechanisms concerned with excitability in cerebral tissues.

Events affected by applying electrical pulses to cerebral tissues are, in likely order of immediacy: ion movements (Cummins & McIlwain, 1961), changes in tissue phosphates (McIlwain & Gore, 1951; Kratzting & Narayanaswami, 1953; Heald, 1958) and changes in respiration and glucose metabolism (McIlwain, 1951; McIlwain, Anguiano & Cheshire, 1952). Action of basic proteins on the latter group of processes has been reported (McIlwain, 1959) and action on tissue phosphocreatine, inorganic phosphate and potassium salts is now described.

EXPERIMENTAL

The conditions, except those in experiments of Table 4, were those recently described (Cummins & McIlwain, 1961) with slices of 80–100 mg. of guinea-pig cerebral cortex prepared and incubated in glucose–sodium hydrogen carbonate media equilibrated with CO₂ + O₂ (5:95). Slices were held in quick-transfer electrodes (Heald & McIlwain, 1956; McIlwain, 1960*b*) during incubation, whether or not electrical pulses were applied; when applied, pulses were alternating, of exponential time–voltage relationship, 10 v peak potential, 0.4 msec. time-constant and at 100/sec. (see McIlwain, 1954). After incubation as described in individual experiments, slices were rapidly transferred to 100 ml. of ice-cold 0.3M-sucrose and from there to 2.5 ml. of 10% (w/v) trichloroacetic acid (Cummins & McIlwain, 1961). In the trichloroacetic acid extracts, phosphocreatine and in-

organic phosphate were determined as described by Woodman & McIlwain (1961), and potassium according to Cummins & McIlwain (1961). After incubation, the potassium content of slices cut first, from the outer convexity of the cortex, is greater than that of slices cut subsequently (Cummins & McIlwain, 1961). Outer slices were included in the experiments of Table 2 but not in those of Table 3; mean potassium values are therefore higher in Table 2. The experiments in Table 4 were carried out in ordinary conical manometric vessels with the glycylglycine–glucose medium and other conditions as described by McIlwain (1959).

Protamine (clupein) sulphate was the analysed specimen of McIlwain (1961*a*); histone sulphate was prepared from calf thymus nuclei (Wolfe & McIlwain, 1961) and suramin was the sodium salt used by Thomson & McIlwain (1961).

RESULTS

Effect of protamine on phosphocreatine and inorganic phosphate

Protamine sulphate at 100 µg./ml. almost completely inhibited the respiratory response of cerebral tissues to electrical pulses (McIlwain, 1959), but in the absence of pulses this and higher concentrations were without effect on tissue phosphocreatine. This is shown in Table 1; in these experiments, slices were incubated throughout in media of the protamine content quoted. At the beginning of incubation the slices contained little phosphocreatine and their inorganic phosphate was high (McIlwain, Buchel & Cheshire, 1951). Consequently the experiments indicate that both the resynthesis and the maintenance of normal concentrations of phosphocreatine are unaffected by the protamine sulphate at 100–400 µg./ml.

Protamine, however, modified the effect of electrical pulses on tissue phosphocreatine. Pulses which normally led to loss of 70–80% of the phosphocreatine in 1–10 min., led with 150 µg. of protamine sulphate/ml. to loss of only 20–40% (Table 1). This action in protamine was nearly maximal at 100 µg./ml.; it resulted in the protamine-treated tissues containing, after pulses for 10 min., 2.5–3 times the phosphocreatine of the corresponding slices without protamine. This is a notable difference, especially as the processes which can yield phosphocreatine, namely respiration and glycolysis, are themselves limited by protamine when pulses are applied.

Inorganic phosphate of the tissues tended to increase with pulses and with protamine, but the changes were not large; reciprocal relationships between phosphocreatine and inorganic phosphate, seen during application of pulses for a few seconds only, are not necessarily to be expected in the much longer period of the present experiments.

Added agents and the potassium salts of the tissue

Tissues that had been incubated in the presence of protamine contained less potassium than those incubated in its absence (Table 2). This action was small at 100 $\mu\text{g.}$ of protamine sulphate/ml., and at its maximum (200–400 $\mu\text{g./ml.}$) involved only 20 % of the tissue potassium. However, Cummins & McIlwain (1961) found that the rapid changes in tissue potassium associated with excitation involved changes of only some 20–30 % in concentration of potassium in the slices and this is also seen to be so in the experiments of Table 2 (first line). At the beginning of incubation in such experiments, tissues were low in potassium content, but stable levels were regained during incubation. Consequently the results of Table 2 (first column) could imply that either a failure to regain potassium, or an increased loss, was caused by protamine.

These processes have therefore been examined separately. Table 2 (second line) shows that when pulses were applied to tissues which had been

incubated with 100 $\mu\text{g.}$ of protamine/ml., the change in tissue potassium followed a similar course to that in normal tissue. Reassimilation of potassium proved, however, to be markedly inhibited by protamine. Table 3 shows, firstly (Expt. 1) the rapid re-entry of potassium observed to occur in normal cerebral tissues (Cummins & McIlwain, 1961) after electrical stimulation had diminished their potassium content by about 10 $\mu\text{equiv./g.}$ The re-entry is seen (Expt. 6) to be greatly inhibited by protamine, such that the change normally occurring in 1 min. is only partly accomplished after 5 min. A histone preparation also inhibited the reassimilation. In this experiment (Table 3, Expt. 5) histone was incubated with the tissue for 90 min. before applying pulses; almost normal reassimilation of potassium followed when incubation was for 30 min. only. This delay in the action of histone was noted earlier in relation to respiratory response to pulses (McIlwain, 1959) and is interpreted as related to diffusion of the histone.

Other circumstances related to re-entry of potassium. The re-entry did not occur when glucose was omitted from the ambient medium. Tissue in such media contained less potassium, but lost the usual quantity on excitation (Cummins & McIlwain, 1961); this was not restored during a 5 min. recovery period (Table 3). In the presence of 2:4-dinitrophenol, a normal content of potassium was reached during preincubation; pulses again

Table 1. *Preservation of phosphocreatine by protamine during application of pulses*

Slices of guinea-pig cerebral cortex (80–100 mg.) were incubated for 40 min. in 5 ml. of glucose–sodium hydrogen carbonate medium. Pulses when applied occupied the last 1 or 10 min. of incubation and were of 10 v peak potential, 0.4 msec. duration and at 100/sec.; they continued until the tissue was plunged into sucrose. It was then rapidly transferred to trichloroacetic acid. Mean values are followed by S.D. where appropriate, and by number of observations (in parentheses).

Protamine sulphate ($\mu\text{g./ml.}$)	Tissue content ($\mu\text{moles/g.}$)					
	No pulses		Pulses (1 min.)		Pulses (10 min.)	
	Phospho-creatine	Inorganic phosphate	Phospho-creatine	Inorganic phosphate	Phospho-creatine	Inorganic phosphate
0	1.50 $\pm$ 0.15 (5)	3.2 $\pm$ 0.2 (5)	0.5 (2)	3.8 (2)	0.32 $\pm$ 0.14 (5)	4.1 $\pm$ 0.5 (5)
100	1.45 $\pm$ 0.26 (5)	3.7 $\pm$ 0.35 (5)	1.0 $\pm$ 0.13 (4)	4.1 $\pm$ 0.4 (4)	0.9 $\pm$ 0.2 (4)	3.7 $\pm$ 0.4 (4)
150	1.50 (2)	4.0 (2)	1.16 (2)	4.0 (2)	0.81 (2)	3.9 (2)
200	1.56 $\pm$ 0.3 (4)	3.6 $\pm$ 0.3 (4)	1.04 (2)	4.3 (2)	0.84 (2)	3.7 (2)
400	1.55 (2)	3.8 (2)	—	—	—	—

Table 2. *Loss of tissue potassium caused by pulses and by protamine*

Guinea-pig cerebral cortex was treated, and results are expressed, as described in Table 1.

Protamine sulphate ($\mu\text{g./ml.}$)	Tissue potassium ($\mu\text{equiv./g.}$)		
	No pulses	Pulses (1 min.)	Pulses (10 min.)
0	61 $\pm$ 4 (8)	56 $\pm$ 3 (8)	47 $\pm$ 4 (8)
100	58 $\pm$ 4 (6)	48 $\pm$ 2 (4)	44.5 $\pm$ 3 (4)
150	53.5 $\pm$ 4.3 (4)	—	45.5 $\pm$ 4 (4)
200	50.5 $\pm$ 1 (4)	—	—
400	50.5 (2)	—	—

Table 3. *Loss and recovery of tissue potassium*

In Expts. 1-7, inner slices of guinea-pig cerebral cortex (80-100 mg.) were incubated in 5 ml. of glucose-sodium hydrogen carbonate medium for 30 min. (Expt. 5 only: for 90 min.), followed by 10 min. during which pulses were applied to the tissues of columns (b), (c) and (d). Tissues (a) and (b) were transferred to sucrose at this point; incubation of tissues (c) and (d) continued as indicated. Transfer and other details: see Table 1. In Expts. 8-10, incubation was as just described, but for 15 min., and was followed by transfer to a second batch of medium, already oxygenated and at 37°, in which the tissue remained for 15 min. before application of pulses and transfer to sucrose as described above.

Group of Expts.	Change in medium	Tissue potassium (μequiv./g.)			
		(a) No pulses	(b) Pulses	(c) Pulses and 1 min. recovery	(d) Pulses and 5 min. recovery
1	None	52 ± 3 (23)	40 ± 3 (18)	47 ± 2 (5)	50 (2)
2	No glucose	39 ± 3 (4)	26 ± 3 (4)	29 (2)	24 (2)
3	30 μM-2:4-Dinitrophenol	51 ± 4 (5)	41 ± 3 (5)	42 (2)	42 (2)
4	Ganglioside, 0.3 mg./ml.	54 ± 3 (4)	44 ± 3 (7)	49 ± 2 (7)	52 (3)
5	Histone sulphate, 0.1 mg./ml.	54 ± 4 (4)	44 ± 1 (4)	46 ± 3 (4)	46 (2)
6	Protamine sulphate, 0.1 mg./ml.	46 ± 4 (14)	40 ± 4 (7)	39 ± 2 (5)	43 ± 2 (7)
7	Protamine sulphate, 0.1 mg./ml., and ganglioside, 0.3 mg./ml.	53 ± 1 (4)	42 ± 4 (4)	50 ± 3 (4)	51 ± 1 (4)
8	First medium: as 6; second: as 1	53 (2)	44 (2)	47 ± 1 (4)	46 ± 3 (4)
9	First medium: as 6; second: as 4	52 (2)	43 (2)	49 (3)	50 (2)
10	First medium: as 6; second, with suramin, 1 mg./ml.	54 (2)	44 (3)	51 ± 3 (4)	51 (3)

Table 4. *Protamine and the stimulated respiration of cerebral tissues*

Manometric vessels contained slices of guinea-pig cerebral cortex of about 60 mg. in 3.5 ml. of glycylglycine-glucose medium in oxygen at 37.5°. Protamine when added was present from the beginning of the experiment. Respiratory rate was measured during 30-40 min. shaking, and then medium or mM-2:4-dinitrophenol (DNP) in medium or M-KCl was tipped from side arms to give the concentrations indicated. Measurement of respiration was continued for a further 30-40 min. Rates quoted are mean values of the number of observations in parentheses, ± S.D. when appropriate.

Protamine sulphate of media (mg./ml.)	Additions (concn. of added substances)				
	None	DNP (30 μM)	DNP (75 μM)	KCl (30 mM)	KCl (60 mM)
	Respiratory rate (μmoles of O ₂ /g. of tissue/hr.)				
0	63 ± 2 (5)	137 (2)	127 (3)	120 (2)	116 (2)
0.1	63 ± 3 (4)	122 (2)	134 ± 6 (4)	75 (2)	81 (2)
0.3	61 (2)	124 (2)	134 (2)	61 (2)	69 (2)

caused loss, but no recovery occurred when pulses were stopped. The concentration of the nitrophenol was chosen as the least needed for a marked effect on respiration and phosphates of cerebral tissues (McIlwain & Gore, 1951). The increase in respiration caused by the dinitrophenol is shown in Table 4 and is not inhibited by protamine, indicating that protamine is not acting directly on respiratory processes; protamine did, however, inhibit the increase in respiration caused by 30-60 mM-potassium chloride, as well as that caused by electrical pulses (McIlwain, 1959).

If after incubation with protamine, tissues were removed from the protamine-containing medium to a normal protamine-free medium (Table 3, Expt. 8), loss of potassium with pulses was readily observed but little re-entry took place after pulses were stopped. This is consistent with the previously observed attachment of protamine to tissues (Thomson & McIlwain, 1961) and its continued inhibition of respiratory response to pulses (McIlwain, 1959).

Gangliosides and suramin. Loss and recovery of potassium were normal in tissues incubated in the presence of gangliosides (Table 3, Expt. 4; these used a ganglioside concentration about that normal to the tissue, and capable of restoring respiratory response to tissues pretreated with protamine: McIlwain, 1959). When tissues were incubated in media containing an inhibitory concentration of protamine together with gangliosides, movements of potassium were again normal (Table 3, Expt. 7). In this situation less protamine becomes attached to the tissue (Thomson & McIlwain, 1961). More significant in appraising the action of gangliosides are Expts. 8-9 of Table 3. Here tissues were preincubated in protamine-containing media and transferred to protamine-free media, some of which contained gangliosides. In this situation protamine remains attached to the tissue (Thomson & McIlwain, 1961); nevertheless, normal recovery of potassium salts occurred. Suramin acted as did gangliosides (Table 3, Expt. 10).

DISCUSSION

Cerebral tissues incubated under the present conditions regain potassium salts from the surrounding medium against a concentration gradient and with the expenditure of metabolically derived energy, as is described quantitatively by McIlwain (1960c) and Cummins & McIlwain (1961); see also Krebs, Eggleston & Terner (1951). The energy requirement is indicated in the present experiments by the lack of potassium re-entry when media lacked glucose or contained 2:4-dinitrophenol and glucose; tissues incubated under either of these conditions contain little or no phosphocreatine (McIlwain & Gore, 1951; McIlwain, 1952) and diminished adenosine triphosphate (Kratzing & Narayanaswami, 1953). Lack of glucose had the more drastic effect on potassium assimilation, for it yielded tissue low in potassium even in the absence of pulses. Presumably 2:4-dinitrophenol allows the formation of energy-rich phosphate ($\sim P$) by glycolysis, and glycolysis increases in its presence until some 100–150 μ moles of lactate are formed/g. of tissue/hr. (McIlwain & Gore, 1951). Increase in tissue potassium after pulses normally proceeds at some 600 μ equiv./g. of tissue/hr., and with a $\sim P$ /potassium ratio of about 1 (Cummins & McIlwain, 1961); it is thus understandable that it failed in the presence of the dinitrophenol. The rate of increase in the potassium content of normal tissues during the present experiments contrasts with that noted in an accompanying paper (Woodman & McIlwain, 1961) to follow the addition of glutamate, this rate being about 20 μ equiv./g. of tissue/hr. and consequently unlikely to make much contribution to the recovery after pulses.

The increase in potassium after pulses represents the utilization of a considerable proportion of the tissue's metabolically derived energy (Cummins & McIlwain, 1961) and is the process now seen to be inhibited by protamine. Moreover, protamine in inhibiting potassium re-assimilation did not diminish phosphocreatine, as did 2:4-dinitrophenol or the absence of glucose: by contrast, protamine preserved phosphocreatine from the diminution usually caused by pulses. Protamine can thus be pictured to be acting on a system which utilizes energy-rich phosphate in ion transport, and this system as operating in tissue as ordinarily incubated, though at a slower rate than after pulses. Thus the lower potassium of the protamine-treated tissues of Table 2 could represent an action on the same process as displayed in Table 3, though only in the latter case are pulses applied.

Restoration of potassium re-assimilation by gangliosides confirms previous conclusions that they can play a role in ion transport (Balakrishnan & McIlwain, 1961; see also Bogoch, 1960). In con-

sidering this role of gangliosides in relation to their chemical nature, it is noteworthy that they are strongly ionized carboxylic acids of equivalent weight and minimum molecular weight about 1,500 (McIlwain, 1961b); they form firm associations with other cerebral constituents (van Heyningen, 1959), and in ground cerebral tissues they occur in membrane-derived fractions (Wolfe, 1961). These properties support a picture of ion transport in which the gangliosides are membrane constituents but are not mobile carriers, constituting rather a hydrophilic path for cation movement through the lipid membrane. A mechanism has been proposed (McIlwain, 1960c) in which gangliosides function at membrane pores of the type envisaged by Danielli (1958), Harris (1960) or Mullins (1959) in association with the sodium- and potassium-activated adenosine triphosphatase present in neural (Skou, 1957, 1960) and cerebral tissues (Hess & Pope, 1957; J. Järnefelt, personal communication; D. H. Deul & H. McIlwain, in preparation.) Protamine could inhibit cation transport in such a pore by combining with acidic groups at its mouth, so interrupting a sequence of negatively charged adsorption sites; and the polyacidic suramin or added gangliosides could restore an acidic environment by masking the basic groups of the protamine while still leaving it attached to the membrane.

SUMMARY

1. Guinea-pig cerebral cortex was maintained *in vitro* and its content of potassium salts and phosphocreatine measured under normal conditions, immediately after applying electrical pulses and during subsequent recovery.

2. Protamine and histone sulphates (0.1 mg./ml.) did not affect the loss of potassium induced by pulses, but inhibited recovery of potassium. This inhibition was removed by gangliosides and by suramin. Higher concentrations of protamine diminished tissue potassium in the absence of pulses.

3. Protamine (0.1–0.4 mg./ml.) was without effect on phosphocreatine in the absence of pulses, but in their presence it preserved a large part of the phosphocreatine from the loss normally caused by pulses.

4. Protamine and histone are considered to act at a mechanism which utilizes a major part of the energy-rich phosphate of the tissue in ion transport, and suggestions are made about the nature of this mechanism.

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Glutamic Acid, other Amino Acids and Related Compounds as Substrates for Cerebral Tissues: Their Effects on Tissue Phosphates

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This investigation was prompted by the apparently opposed actions shown by glutamic acid in cell-free and in cell-containing systems derived from mammalian brain.

When cell-containing cerebral tissues respire in glucose media, phosphocreatine is resynthesized from creatine already present in the tissue; addition of glutamic acid then increases respiratory rate (Weil-Malherbe, 1936) but decreases phosphocreatine (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1952). When added as only oxidizable substrate, glutamic acid again supports relatively high respiratory rates but does not lead to re-synthesis of phosphocreatine. Nevertheless, with ground cerebral tissues or with mitochondrial preparations from them, glutamic acid supports oxidative phosphorylation (Brody & Bain, 1952), and thus would be expected to lead to the accumulation of energy-rich phosphates including phosphocreatine.

One interpretation of these findings is that they indicate the occurrence of additional, energy-consuming processes in the cell-containing tissue, processes which are diminished with loss of cell structure or not represented in the mitochondrial

systems. Thus when it was observed that the addition of glutamic acid to tissues respiring in glucose increased the quantity of potassium salts associated with a given weight of tissue (Terner, Eggleston & Krebs, 1950), the assimilation of potassium salts appeared likely to represent the additional energy-consuming process. Pappius & Elliott (1956) have since shown that the additional potassium under these conditions is associated with increased volume, the concentration of potassium salts falling or remaining little changed.

In the present study, other processes which might lead to loss of adenosine triphosphate or phosphocreatine in the presence of glutamate have been examined. These include the synthesis of glutamine, noted by Krebs (1935) and Weil-Malherbe (1936), the appearance of inorganic pyrophosphate from reactions such as those described with amino acids by Moldave, Castelfranco & Meister (1959) and the displacement of creatine from the tissue. In addition, evidence has been sought by comparing the action of glutamic acid on tissue phosphates with the actions of other amino acids and with related keto and guanido derivatives.