Guanosine Di- and Tri-phosphates in the Phosphate Metabolism of Cerebral Tissues Promoted by Electrical Pulses

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In a previous communication (Heald, 1957*b*) it was shown that the 'phosphoprotein' fraction of cerebral tissues was implicated in the rapid phosphate-exchange reactions promoted by applied electrical pulses (Heald, 1956*b*). It was pointed out that owing to the crudeness of the analytical methods employed the nature of the phosphoprotein could not be determined, more especially since its estimation depended upon a preliminary degradation to inorganic phosphate. It was also considered that since the phospholipid fraction showed no change in specific radioactivity upon the passage of pulses, the phospholipids were not involved in the rapid-exchange reactions which were described. However, it was pointed out by Dr G. H. Sloane-Stanley and subsequently by Professor J. Folch that treatment of the tissue with trichloroacetic acid to remove phosphates soluble in this extractant before further fractionation would affect the extractability of the phospholipids, some of which might conceivably remain unextracted and be degraded to inorganic phosphate during the analysis for 'phosphoprotein'.

For these reasons attempts were made to isolate from cerebral tissues, under conditions less degradative than those previously used, a phosphate fraction or fractions which increased in specific radioactivity when electrical pulses were applied for 10 sec. to tissue which had metabolized radioactive phosphate for a brief period. Such an increase in specific radioactivity under these conditions had previously been found to occur with the phosphoprotein fraction.

This has led to the discovery that guanosine di- and tri-phosphates are components of cerebral tissue implicated in this type of response. A preliminary account of this work was presented to the Biochemical Society in November 1956 (Heald, 1957*a*).

MATERIALS AND METHODS

Tissues, media and general methods of analysis and apparatus

These have been described in detail in previous publications (Heald, 1956*a, b*, 1957*b*) together with techniques of manipulation of the cerebral slices. Only procedures specific to the work described here are given.

Fixation and extraction of cerebral slices

Slices were fixed either in 5 ml. of CHCl_3 -methanol (2:1, v/v) (Folch, Ascoli, Lees, Meath & LeBaron, 1951), at -15° , or in saturated $(\text{NH}_4)_2\text{SO}_4$ at 0° buffered to pH 4 with 0.1 M-sodium acetate (Lowry & Lopez, 1946). They were disintegrated in small glass tubes with a fitting pestle and centrifuged at 25 000 *g* in the superspeed head of the M.S.E. Major centrifuge for 15 min. The temperatures of centrifuging were -15° for CHCl_3 -methanol extracts and 0° for $(\text{NH}_4)_2\text{SO}_4$ extracts.

Tissues fixed in ammonium sulphate. The residues remaining after extraction were digested for the determination of total phosphate and radioactivity. The $(\text{NH}_4)_2\text{SO}_4$ supernatants were analysed for inorganic phosphate as described by Lowry & Lopez (1946) with the addition of CuSO_4 to a final concentration of 4×10^{-5} M to increase the rate of reaction (Peel, Fox & Elsdon, 1955). Phosphocreatine was determined in these extracts as the difference between inorganic phosphate and the total phosphates extractable into isobutanol after incubation of an extract diluted five times with acid molybdate for 30 min. at 25° . Dilution of the extracts was essential, for above 20% saturation with $(\text{NH}_4)_2\text{SO}_4$ the phosphomolybdate complex was not extracted by isobutanol.

Tissues fixed in chloroform-methanol. After extraction with at least 20 vol. of CHCl_3 -methanol (2:1, v/v) the residues were washed once with ethanol at -15° , and extracted at 0° four or five times with 0.5 M-2-amino-2-hydroxymethylpropane-1,3-diol (tris) saturated at pH 7.4 with $(\text{NH}_4)_2\text{SO}_4$. The ethanol wash was essential to remove CHCl_3 from the residue, which otherwise formed an unmanageable gum when $(\text{NH}_4)_2\text{SO}_4$ was added. The residues were extracted three times with aq. NH_3 soln. (0.28 M), pH 10.5–11.0, at 0° , a fluid:tissue ratio of 0.5 ml.:100 mg. original wet wt. of tissue being used. This brought the bulk of the radioactive material into solution. The radioactive phosphates in this extract were further fractionated upon a column of Dowex-1 chloride either 4 or 8% cross-linked.

Treatment of the chloroform-methanol extracts

To remove contaminating water-soluble phosphates the extracts were washed first with 0.2 vol. of 0.03 M-NaCl and then five times with 0.2 vol. of the upper aqueous phase of an equilibrated mixture of 5 parts of CHCl_3 -methanol (2:1, v/v) and 1 part of 0.03 M-NaCl. This mixture was considered to correspond to that produced by the addition of 0.2 vol. of NaCl solution to the original extracts. Unless NaCl is included the aqueous layers contain acidic phospholipids and sulphatides and in addition tend to form unmanageable

gels. The addition of NaCl prevents this (Folch, Lees & Sloane-Stanley, 1954). After washing, samples of the CHCl_3 extract were taken for the determination of total phosphorus and radioactivity.

Fractionation of the ammoniacal extract on Dowex-1 chloride

For experiments involving 800 mg. of tissue, a column $1 \text{ cm.}^3 \times 3 \text{ cm.}$ was used. The ammoniacal extract was diluted 10–20 times with water, to reduce the concentration

of $(\text{NH}_4)_2\text{SO}_4$ to below 0.01 M , and was passed through the resin at the rate of 3 ml./min. Usually all the radioactivity was absorbed. If counts appeared in the effluent from such a column it was attributed to insufficient dilution. When all the extract had been added the column was washed with $50\text{--}100 \text{ ml.}$ of water and eluted with 0.005 N-HCl , 0.01 N-HCl and 0.1 N-HCl containing $0.5 \text{ M-NH}_4\text{Cl}$. Two fractions A and B were obtained (Fig. 1a). The choice of eluents was made on the basis of preliminary experiments with the gradient-elution technique with differing initial concentrations of HCl and NH_4Cl .

Analysis of fractions A and B for total phosphate and radioactivity was accomplished by evaporating almost to dryness and digesting the residues as described previously (Heald, 1956a). The residue of fraction B was first heated to remove excess of NH_4Cl since high concentrations of this interfere with the extraction of the phosphomolybdate complex.

Purification of fraction B. Further purification of fraction B was achieved by adsorption on to acid-washed charcoal (British Drug Houses Ltd.) and elution with aq. 0.01 M-NH_4 soln.– 0.1 M-phenol . Before elution, the column was washed with water until the effluent no longer gave any reaction with barium acetate solution. Phenol was removed from the eluate by extraction with ether and the aqueous solution was freeze-dried. The products, fractions C, were pale straw-coloured powders, free from chloride and sulphate.

The whole procedure is outlined in Fig. 2.

Preparation of fraction C from ox brain

Ox brain was obtained from an abattoir (Croydon Abattoir, 33 Cuthbert Road, Croydon), and was removed whole and placed in crushed solid CO_2 within 3–4 min. of the death of the animal. This was found to be necessary since the quantity of the fraction sought decreased to one-fifth of the level found in frozen brain if the tissue was allowed to remain at air temperature during the 1–1.5 hr. required for transport to the laboratory. In a typical experiment the brain (320 g.) was broken while frozen into portions of 38 g. and each portion was extracted in a Waring Blender with 760 ml. of ice-cold CHCl_3 –methanol (2:1, v/v) for 3 min. The suspension was filtered and the residue washed on the filter with 200 ml. of ethanol at -15° . The washed residue was extracted in the Blender with saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.4, 0.5 M-tris), 200 ml. of solution being used/10 g. of residue. This procedure resulted in some rise in temperature and required speed of operation. The suspension was filtered at $+2^\circ$, this being simpler than centrifuging from such a dense medium. The residue was then extracted in the Blender with aq. 0.28 M-NH_4 soln., 200 ml./10 g. of residue. The extracts were centrifuged, combined and diluted to 10 l. To this was added, as a marker, a radioactive extract prepared from guinea-pig cerebral tissue which had been allowed to metabolize ^{32}P under the conditions used in this and previous studies (Heald, 1956b). The diluted total extract was divided into two portions and each was passed, under pressure, through a column of Dowex-1 chloride $1.8 \text{ cm.}^3 \times 15 \text{ cm.}$ This operation was conducted in the cold room to minimize breakdown of labile phosphates during the long period necessary to pass the solutions through the resins. Dividing the extract as above enabled the whole 10 l. to be passed through overnight. Fraction B was then eluted as described above, the radioactivity of the marker

Fig. 1. Fractionation of radioactive phosphates in ammoniacal extracts of cerebral tissues upon Dowex-1 chloride. Column $1 \text{ cm.}^3 \times 3 \text{ cm.}$ Fractions A and B were eluted with 0.005 N-HCl and 0.1 N-HCl containing $0.5 \text{ M-NH}_4\text{Cl}$, respectively. (a) Fractionation of an extract prepared from tissue which had metabolized radioactive phosphate; (b) fractionation of an inactive extract to which radioactive orthophosphate had been added. Fraction volumes were 10 ml. in all cases.

Fig. 2. Fractionation of cerebral tissue to obtain phosphates increasing in specific radioactivity when derived from slices metabolizing radioactive phosphate and subjected to electrical pulses.

Fig. 3. Separation of three fractions from fraction B (see Figs. 1 and 2) by adsorption on charcoal and elution with aq. 0.014 M-NH₃ soln., 1% (w/v) phenol. Fraction C₁ was not adsorbed on the column. The column consisted of acid-washed charcoal, 1.8 cm.² × 15 cm., 10 ml. fractions being collected.

sample indicating the point at which the fraction was removed from the column.

Fraction B was refractionated on charcoal columns 1.8 cm.² × 15 cm. containing about 10 g. of charcoal.

A typical fractionation is shown in Fig. 3. Two major fractions were usually obtained, designated C₂ and C₃ respectively, fraction C₁ being that not adsorbed on charcoal. After removal of phenol, the eluates were freeze-dried.

Analytical methods

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with a 1:250 dilution of serum as an arbitrary standard.

Pentose. This was estimated according to the procedure of Albaum & Umbreit (1947), readings of the colour intensity being taken at 7, 20 and 45 min. respectively to distinguish between the pentose 3'- and 5'-phosphates.

Paper chromatography. This method was used for the separation of guanosine and adenosine di- and tri-phosphates. The solvent was propan-1-ol-aq. NH₃ soln.-water (60:30:30, by vol.). In this solvent adenyl derivatives run ahead of guanyl derivatives and the two are separable in 18-24 hr. Purines were separated from hydrolysates of the nucleotides (1 hr. in N-HCl at 100°) by chromatography in propan-2-ol-HCl (Wyatt, 1951). They were detected in ultraviolet light from a Chromatolite lamp (Hanovia Ltd., Slough, Bucks) and eluted in 0.1 N-HCl for determination of the absorption spectra.

Paper electrophoresis. Nucleoside di- and tri-phosphates were separated by paper electrophoresis in 0.05 M-citrate buffer, pH 3.5.

RESULTS

The choice of conditions for extraction was determined by the criterion that the specific radioactivity of the fractions examined increased when these were

Table 1. *Effect of electrical pulses on the distribution of radioactive phosphorus in fractions of cerebral tissue initially denatured in chloroform-methanol (2:1, v/v) at -15°*

After denaturation the tissues were extracted with saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 7.4. Conditions as in the Methods section. Values given are the specific radioactivities of the phosphorus of each fraction. Different amounts of radioactive phosphorus were used in different experiments. Pulses, condenser pulses 18v peak potential, 0.4 msec. duration, were applied for 10 sec. only.

Expt. no.	CHCl ₃ -methanol extract			Residue after $(\text{NH}_4)_2\text{SO}_4$ extraction		
	With pulses	Without pulses	Difference	With pulses	Without pulses	Difference
1	0.52	0.63	-0.11	5.40	3.00	+2.40
2	0.04	0.04	0.0	5.85	3.16	+2.69
3	0.28	0.30	-0.02	17.4	9.65	+7.75
4	3.12	1.40	+1.72	43.5	38.0	+5.50
5	4.70	2.54	+2.16	59.0	46.0	+13.0
6	0.82	0.45	+0.37	5.25	4.4	+0.85
7	0.69	0.62	+0.07	25.6	24.5	+1.10
8	1.53	1.44	+0.09	42.0	32.7	+9.3
9	—	—	—	26.4	14.3	+12.1

$P < 0.2 > 0.1$

$P < 0.01$

derived from cerebral slices incubated in the presence of radioactive phosphorus and subjected to electrical pulses. It was also required that such fractions were free from contaminating inorganic phosphate, phosphocreatine and adenosine triphosphate.

Experiments in which the tissue was fixed in ice-cold saturated $(\text{NH}_4)_2\text{SO}_4$, pH 4 (Lowry & Lopez, 1946), failed to demonstrate that such a change in specific radioactivity occurred in the tissue residues. It was found that if the phospholipids were removed by initially fixing the slice in CHCl_3 -methanol (2:1, v/v) (Folch *et al.* 1951) and subsequently washing the residue with saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.4) at 0°, the washed residue increased in specific radioactivity if pulses had been passed through the original tissue slice (Table 1). No significant increase was noted in the activity of the phospholipid fraction.

Determination of phosphocreatine and inorganic phosphate in the extracts showed that they were quantitatively removed from the tissue. Contaminating radioactive inorganic phosphate and other water-soluble phosphates were removed from the CHCl_3 -methanol extract by washing as described in the Methods section. This reduced contamination by added inorganic phosphate to 0.1%. Similarly, extraction of the non-phospholipid residue with saturated $(\text{NH}_4)_2\text{SO}_4$ reduced contamination to 0.3-0.5% of the amount of orthophosphate originally added. Further extractions did not reduce the degree of contamination any more and this constituted an appreciable error if quantities in excess of 4-5 μC of ^{32}P were added to each tissue slice. Contamination from this quantity was calculated to alter the specific radioactivity of the final residue by 0.3-0.5 unit only. The third and fourth $(\text{NH}_4)_2\text{SO}_4$ extracts did not absorb at 260 $\text{m}\mu$,

indicating that all extractable nucleotides had been removed.

Adenosine triphosphatase activity of the tissue was absent during fixation. Thus for tissue fixed in CHCl_3 -methanol in the presence of 0.771 μmole of adenosine triphosphate the levels of inorganic phosphate were 0.17 ± 0.02 (6) μmole of P/100 mg. of slice, and for tissues fixed in the absence of adenosine triphosphate, inorganic phosphate levels were 0.13 ± 0.02 (4) μmole of P/100 mg. of slice. No activity was detectable in the tissue residue after extraction of the phospholipids.

Extraction of the non-phospholipid residue

Since previous work (Heald, 1957*b*) had suggested that the required fraction was a 'phosphoprotein', most use was made of the extraction at alkaline pH. In Table 2 is shown the percentage activity extracted from the residue by solutions of different pH. As the pH of the extractant increased, the percentage of radioactivity in the extract increased. Above pH 6.8 the quantity of protein extracted paralleled the radioactivity of the extract (Table 2, column 3), and it was therefore considered that the same material was being extracted in increasing quantity. Since the material sought was thought to be labile in alkali, solutions above pH 11.5 were not used. Ammonia solution was the extractant of choice since it could be easily removed.

The specific radioactivity of the ammoniacal extract increased when pulses were applied to the tissue slices. Inorganic phosphate, as estimated by the method of Berenblum & Chain (1938), also increased in specific radioactivity. No significant change was noted in the extracted residue (Table 3). The increase in the specific radioactivity of the inorganic phosphate fraction was contrary to that expected from previous results (Heald, 1956*b*) and

proved very difficult to assess for the following reasons. Direct application of the Berenblum & Chain technique to the ammoniacal extracts, containing added radioactive inorganic phosphate, failed to extract all the added phosphate. This was due to the formation of an opaque layer at the interphase of the *isobutanol* and the acidified extract. This layer contained neither radioactivity nor phosphorus until molybdate was added to the acid solution, when the layer became radioactive. Presumably the phosphomolybdate complex was partially adsorbed by the layer. Inclusion of this layer with the *isobutanol* extracts led to correct values for radioactivity but gave high values for inorganic phosphate. Thus in these experiments the levels of inorganic phosphate in solution were, by the Berenblum & Chain procedure, 1.07 ± 0.15 (3) $\mu\text{mole/g. wet wt.}$ of original tissue, and by the Lowry & Lopez (1946) method 0.64 ± 0.12 (3) $\mu\text{mole/g. wet wt.}$ The Lowry-Lopez method is generally considered to yield a correct value for inorganic phosphate in solution. The quantity of inorganic phosphate found could not arise by contamination or adsorption of the phosphate of the tissue slice (normally $1.5\text{--}2.0$ $\mu\text{moles/g. wet wt.}$), since such contamination would not exceed 0.5% (see above).

Attempts to separate inorganic phosphate and other phosphates in the extracts, by freeze-drying and subjecting the residue to paper electrophoresis, were unsuccessful. All the fractions obtained were shown by control experiments to contain inorganic phosphate as a contaminant. Trichloroacetic acid at

a final concentration of 10% (w/v) did not precipitate any radioactive component.

It was found possible to separate inorganic phosphate and a metabolically active phosphate fraction by chromatography on Dowex-1 chloride. A typical fractionation of extracts from 1 to 2 g. of guinea-pig cerebral slices is shown in Fig. 1 (a), and in Fig. 1 (b) is shown the fractionation of an inactive extract to which radioactive inorganic phosphate had been added. Clearly the fraction B did not contain inorganic phosphate. Fraction A was not always retained by the resin, particularly if the quantity of ammonia in the extracts was increased. Thus continued washing with aq. $0.02\text{--}0.03\text{M-NH}_3$ soln., such as occurred if much extract was to be fractionated, brought most of fraction A off the column before elution with HCl was begun. Possibly inorganic phosphate was bound to some basic protein or peptide in the mixture.

Fractions A and B together accounted for $80\text{--}90\%$ of the total radioactivity added to the column, less than 3% being retained after elution of fraction B. In terms of phosphorus these fractions accounted for only 50% of the total phosphorus present in the extract. Thus the ammoniacal extracts contained 2.21 ± 0.17 (12) $\mu\text{moles of P/g. wet wt.}$ of tissue whereas fractions A and B contained 0.56 ± 0.1 (9) and 0.68 ± 0.12 (8) $\mu\text{mole of P/g. wet wt.}$ respectively. The discrepancy is presumably due to inactive phosphates or phosphates of low radioactivity which were not eluted by 0.1N-HCl , $0.5\text{M-NH}_4\text{Cl}$.

Table 2. *Extraction of radioactive phosphate material and protein from the non-phospholipid residue of cerebral tissue by various extractants*

Residues were shaken with extractant, 0.5 ml. of extractant/ 100 mg. original wet wt. of tissue being used, at $0\text{--}2^\circ$ for periods of 5 min. Extractions were repeated until little more radioactivity appeared in the extracts.

Extractant	pH	Counts in solution as percentage of the counts originally present	Protein extracted as percentage of the total originally present	Ratio: % protein % counts
Acetate (0.05M)	4.0	32.0 ± 6.0 (3)	3.61 ± 0.8 (3)	0.129
Tris (0.05M)	6.8	41.0 ± 4.4 (3)	10.8 ± 1.1 (3)	0.263
Tris (0.05M)	9.0	57.1 ± 4.4 (3)	13.5 ± 0.6 (3)	0.24
Ammonia soln. (0.28M)	11.5	78.5 ± 6.0 (6)	19.9 ± 2.0 (3)	0.258

Table 3. *Effects of electrical pulses on the distribution of radioactivity in ammoniacal extracts of guinea-pig cerebral slices, after removal of phospholipids*

Conditions of incubation of tissue were as given in the Methods section. Pulses, 18V peak potential, 0.4 msec. duration, were applied for 10 sec. Values are specific radioactivities (counts/min./ $\mu\text{g. of P}$) $\pm$ S.E.M. Figures in parentheses are the number of determinations.

Fraction					
Total P in ammonia extract		Orthophosphate in extract		Residual P	
With pulses	Without pulses	With pulses	Without pulses	With pulses	Without pulses
45.1 ± 3.6	18.0 ± 1.97	56.7 ± 4.0	29.4 ± 5.5	8.28 ± 1.14	6.47 ± 0.84
(3)	(3)	(3)	(3)	(3)	(3)

Table 4. *Effect of electrical pulses on the specific radioactivity of two phosphate fractions obtained from cerebral slices after incubation in saline containing radioactive inorganic phosphate*

Conditions were as described in the Methods section. Fractions *A* and *B* refer to the fractions depicted in Fig. 1*a*. Differing amounts of radioactivity were used in different experiments. Condenser pulses 18 v peak potential, 0.4 msec. duration, were applied for 10 sec.

Expt. no.	Specific radioactivity of the fraction				
	Fraction <i>A</i>		Fraction <i>B</i>		
	With pulses	Without pulses	With pulses	Without pulses	Difference
1	565	692	178	118	+60
2	547	517	112	88	+24
3	852	745	226	80	+146
4	1120	960	196	90	+106
<i>P</i> < 0.001					

The effect of electrical pulses upon the specific radioactivity of fractions *A* and *B* is shown in Table 4. When applied for a period of 10 sec., pulses induced an increase averaging 95% in the activity of fraction *B* and a smaller but variable increase in fraction *A*. The difference between the individual experiments of Table 4 is probably due to the different quantities of radioactive phosphate used.

That the phosphate in fraction *B* was metabolically incorporated was shown by two types of experiment. In the first, radioactive phosphate was added to slices metabolizing in a glucose medium, 10 sec. before fixation. During this period pulses were passed. Fractionation on Dowex-1 revealed a pattern similar to that of Fig. 1(*b*). This experiment would appear to rule out the increase in activity being due to permeability changes induced by applied pulses. In the second experiment glucose was omitted from the medium, but otherwise the experiment was performed in a manner identical with that yielding the results in Fig. 1(*a*). Here also pulses were applied during the last 10 sec. before fixation. The specific radioactivity of fraction *B* was 0.17–0.20 of that obtained in experiments where glucose was present.

Removal of salts from fraction *B*. As obtained from the Dowex column fraction *B* contained a high concentration of NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$. These were removed by adsorbing the fraction on charcoal and eluting with phenol-aq. NH_3 soln. (see Fig. 3). A small amount (12%) was not adsorbed and about 4% was retained by the column. Recovery of phosphorus was almost quantitative. Thus in three determinations the amounts added to the column were 0.60, 0.44 and 0.5 μmole of P/g. wet wt., and the amounts recovered were: (*a*) not absorbed, 0.003, 0.02 and 0.02 μmole of P/g. wet wt.; (*b*) as the major peak, 0.57, 0.36 and 0.42 μmole of P/g. wet wt. respectively.

Identification of the components of fraction B

Ox brain was obtained and fractionated as described in the Methods section. The peaks labelled C_2 and C_3 (see Fig. 3) were examined separately. Paper chromatography in propan-1-ol-

aq. NH_3 soln.-water revealed that each fraction contained at least two components designated $C_{2(1)}$, $C_{2(2)}$ and $C_{3(1)}$, $C_{3(2)}$, for the slowest- and fastest-running components respectively. From the positions of the spots on guide strips suitable sections of larger chromatograms were cut out and eluted with water. Each eluate upon paper electrophoresis at pH 3.5 was further resolved into two components, $C_{2(1)a}$, $C_{2(1)b}$, etc., subscripts *a* and *b* representing the slower- and faster-moving components respectively.

Fractions $C_{2(1)}$ and $C_{3(1)}$ contained only guanine, whereas fraction $C_{3(2)}$ contained only adenine. Fraction $C_{2(2)}$ contained adenine and guanine. Fractions $C_{2(2)a}$, $C_{2(2)b}$ were lost after electrophoresis but, from the position of the marker spots and subsequent analysis of the other fractions, were considered to be adenosine diphosphate and guanosine triphosphate respectively.

The time course of development of orcinol colour during the determination of pentose was identical with that of an adenosine 5'-phosphate (Albaum & Umbreit, 1947). This analysis was carried out either on the single components or, as in fraction $C_{2(2)}$, upon the mixture. The absorption spectra of the orcinol colours were identical with that obtained from adenosine 5'-triphosphate, indicating that hexoses were absent. It is considered that the components were pentose 5'-phosphates, the pentose probably being ribose. Analysis of the individual phosphates and suggested identity is given in Table 5.

In Table 6 is shown the effect of pulses upon the distribution of radioactivity in the main fractions described above. Clearly the increase in specific radioactivity took place in those fractions containing guanosine nucleotides. In all nucleotides the radioactivity was contained in the β - and γ -phosphorus atoms, since all radioactivity was liberated as inorganic phosphate upon hydrolysis for 10 min. at 100° in $\text{N-H}_2\text{SO}_4$. The activity of the individual fractions listed in Table 5 was not estimated under these conditions, for the quantities of nucleotides obtained from 100 g. of brain after processing as

Table 5. *Analysis of nucleotides of fraction B obtained from ox brain by separation of ammoniacal extracts on Dowex-1 chloride, and on charcoal followed by paper chromatography and electrophoresis*

For details see Methods section and text. Values are given as $\mu\text{mole/ml.}$ of sample used.

Details of separation	Fraction	Identity of base	Base	Total P	Labile P	Pentose	Proposed identity
Separated in propanol-aq. NH_3 soln.-water (60:30:30, by vol.)	$C_{2(1)}$	Guanine	0.68	1.20	0.71	0.68	Guanosine diphosphate (GDP)
	$C_{2(2)}$	Guanine and adenine	0.45	1.10	0.71	0.42	Mixture of guanosine triphosphate (GTP) and adenosine diphosphate (ADP)
Separated by electrophoresis at pH 3.5	$C_{3(1)}$	Guanine	0.26	0.55	0.25	0.19	Guanosine di- and tri-phosphates
	$C_{3(2)}$	Adenine	0.88	1.55	0.93	0.68	Adenosine di- and tri-phosphates
	$C_{3(1),a}$	Guanine	0.11	0.24	0.11	0.10	Guanosine diphosphate
	$C_{3(1),b}$	Guanine	0.10	0.30	0.17	0.09	Guanosine triphosphate
	$C_{3(2),a}$	Adenine	0.40	0.79	0.42	0.40	Adenosine diphosphate
	$C_{3(2),b}$	Adenine	0.32	0.85	0.50	0.28	Adenosine triphosphate (ATP)

Table 6. *Effect of electrical pulses on the distribution of radioactivity in nucleotides isolated from guinea-pig cerebral slices and ox brain*

Nucleotide fractions were isolated from ox brain to which marker fractions, prepared from slices of guinea-pig cerebral cortex, had been added. For details see Methods section and text. Values are given as radioactivity of the phosphates (counts/min./ $\mu\text{g.}$ of P). Pulses, 18v peak potential, 0.4 msec. duration, were applied to slices furnishing the marker fractions for 10 sec.

Fraction	Identity*	Counts/min./ $\mu\text{g.}$ of P	
		With pulses	Without pulses
B	Mixed nucleotides	34.2	28.6
C_2	GDP, GTP, ADP	32.6	28.8
C_3	GTP, GDP, ADP, ATP	35.5	30.0
$C_{2(1)}$	GDP	20.1	16.0
$C_{2(2)}$	ADP, GTP	54.0	42.0
$C_{3(1)}$	GDP, GTP	40.0	26.8
$C_{3(2)}$	ADP, ATP	45.0	46.0

* See Table 5.

described above was small, and it became difficult to accumulate marker fractions of sufficient activity to be of use with larger quantities of brain. Also the length of time involved in working up the material further reduced the activity of the fractions.

DISCUSSION

Experiments detailed above were directed towards the isolation, from cerebral slices metabolizing radioactive inorganic phosphate, of phosphates which increased in specific radioactivity upon brief passage of electrical pulses through the slices. In this respect the experiments, while yielding a positive result in the finding of guanosine triphosphate as such a component, nevertheless yielded mainly negative results with respect to further characterization of the 'phosphoprotein' fraction originally sought.

The presence of guanosine di- and tri-phosphate in brain was first indicated by Kerr (1942), but no further evidence was obtained until Schmitz, Hurlbert & Potter (1954) briefly mentioned its presence in extracts of rat brain. More recently Thomas (1957) has found that nucleotides from extracts of guinea-pig cerebral slices yield guanine amounting to $0.3 \mu\text{mole/g.}$ wet wt. upon acid degradation. The identification of guanosine di- and tri-phosphate as cerebral nucleotides, described here, confirms Kerr's earlier deduction. The quantities of guanosine derivatives, $0.2-0.3 \mu\text{mole/g.}$ wet wt., found in the ammoniacal extracts constitute the total amount found by Thomas (1957) in guinea-pig brain, suggesting that guanosine di- and tri-phosphate are not extracted by the methods used above. This conclusion is rendered more

likely by failure to find guanosine in hydrolysates of the nucleotides precipitated from the ammonium sulphate extracts by the method of Heald & Long (1955). The quantity of guanosine derivatives relative to the total nucleotides present is much larger in brain than in muscle. Thus whereas in rat muscle guanosine triphosphate constitutes only 2–3 % of the total nucleotides present (Bergkvist & Deutsch, 1953; Threlfall, 1957), in brain guanosine phosphates account for 18–20 % of the total nucleotides both *in vivo* and *in vitro*. It seems reasonable to assume that in nervous tissue guanosine derivatives take part in some function more specific to the tissue than to non-nervous tissue. As isolated from ox brain the nucleotide fraction contained 0.26 μ mole of adenine nucleotides/g. wet wt. or approx. 20 % of the total normally present in cerebral tissues *in vitro* (Heald, 1956*a*). Whether these adenine nucleotides are present as contaminants due to difficulty in washing large quantities of cerebral extracts with saturated ammonium sulphate, or whether they constitute nucleotides metabolically distinct from those extracted by the ammonium sulphate, is not known. Experimental arrangements other than those described above would be needed to investigate this more fully.

The role of guanine nucleotides in a phosphate-transfer reaction of the type previously envisaged (Heald, 1956*b*) cannot be decided from the present evidence. Recently (Ayengar, Gibson, Peng & Sanadi, 1956), it has been shown that guanine nucleotides are involved in the phosphorylation of adenosine diphosphate to adenosine triphosphate during the breakdown of succinyl-coenzyme A. If such transphosphorylation occurs in cerebral tissue it must be the reverse of that described by Ayengar *et al.* for the specific radioactivity of guanine nucleotides increased on passing electrical pulses, whereas that of the adenine nucleotides decreases under similar conditions (Heald, 1956*b*).

Failure to obtain clear evidence of the presence of a phosphoprotein fraction also involved in the phosphate-exchange reaction can probably be attributed to the different conditions used. Previous demonstrations of the participation of phosphoprotein in the exchange (Heald, 1957*b*) were carried out upon tissue degraded in trichloroacetic acid, whereas the present experiments were carried out under alkaline conditions. It is possible that the phosphoprotein, demonstrated in tissue degraded under acid conditions, is in fact a stable degradation product of a more labile phosphate which is split to other products under alkaline conditions. Such a possibility has been considered by others (see, for example, Perlman, 1955) and it is of interest to note

that, in so far as the determinations permitted, the inorganic phosphate found in the ammoniacal extracts of cerebral slices (Table 3) increased in specific radioactivity upon passage of pulses.

SUMMARY

1. Following upon a previous study, attempts have been made to isolate, from guinea-pig cerebral slices metabolizing radioactive inorganic phosphate *in vitro*, phosphates which increase in specific radioactivity upon the passage of electrical pulses through the slices.

2. A fraction possessing this property has been shown to consist of adenosine and guanosine di- and tri-phosphates, which were isolated and characterized. Only the guanine nucleotides increased in specific radioactivity upon the passage of pulses.

3. No other fraction was found which showed such an increase. It is suggested that the cerebral phosphoprotein fraction previously shown to increase in specific radioactivity under similar conditions is a stable degradation product of a more labile compound which is split to other products, possibly including inorganic phosphate, under the alkaline conditions used in the present experiment.

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