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Analysis of Radioactive Phosphates in Extracts of Cerebral Tissues

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As a means of studying the metabolism of radioactive phosphates in extracts of cerebral tissue, a method of analysis was required applicable to the fractionation of small quantities (120–130 μg. of total P), and specific for adenosine triphosphate, inorganic orthophosphate and creatine phosphate; it was also desirable that it should be capable of easy extension to a survey of other compounds or groups of compounds.

The methods in general use for the analysis of phosphorylated intermediates, though of considerable value, suffer not only from the relatively large amounts of tissue necessary but also from the inherent defect of overlapping of fractions (Greenberg, 1952). This, though small, becomes important when studies of the distribution of radioactive phosphates is to be considered (Abood & Gerard, 1952; Ennor & Rosenberg, 1952; Lee & Eiler, 1953; Sacks, 1949). To overcome these difficulties it was decided to try preliminary fractionation of phosphates into groups by means of barium followed by paper chromatography.

The results of Turba & Enenkel (1951), Maurer & Schild (1951), and Schild & Bottenbruch (1953) suggested that ionophoresis on paper would achieve a rapid and complete separation of the phosphates, and studies were commenced along these lines. Recent work (Neil & Walker, 1954; Wade & Morgan, 1954, 1955), has shown the separation of phosphates by paper ionophoresis, but the application to the fractionation of tissue extracts has not been reported.

MATERIALS AND METHODS

Adenosine triphosphate (*ATP*). Three samples of ATP were used. Preparation 1 was prepared from the dibarium salt supplied by Boots Pure Drug Co., by means of the ion-exchange resin Deacidite FF (Permutit Co. Ltd.) with the HCl–NaCl eluants recommended by Cohn & Carter (1950). ATP was precipitated as the barium salt from the fraction removed by 0·2 M-NaCl–0·01 N-HCl and after conversion into the potassium salt with potassium sulphate was stored in solution at −20°. The solution showed a sharp symmetrical absorption curve with a peak at 260 mμ. The acid-labile phosphorus determined by 10 min. hydrolysis in N-H_2SO_4 was 68% of the total phosphorus. A preparation of rabbit-muscle myosin liberated 43% of the labile P in 1 hr. and 43·5% in 1·5 hr. When subjected to paper ionophoresis at pH 4·3 the solution showed only a single phosphate-containing compound. Inorganic orthophosphate was absent. The product was considered to be pure ATP.

Preparation 2 was prepared from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). The final product contained 64% acid-labile phosphorus, and it migrated mainly as a single spot on ionophoresis at pH 4·3. A faint spot corresponding to adenosine diphosphate (ADP) was present. Inorganic orthophosphate was absent.

Preparation 3 was a chromatographically pure sample of the crystalline sodium salt, and was a gift from Sigma Chemical Co., St Louis, U.S.A.

Adenosine diphosphate (ADP) was also a gift from Sigma Chemical Co. of the chromatographically pure sodium salt.

Creatine phosphate was prepared from creatine by the method of Ziele & Fawaz (1938). The product obtained as the calcium salt was stored over P_2O_5.

Glucose 1-phosphate was a gift of the pure dipotassium salt from Roche Products Ltd. The material migrated as a single spot on ionophoresis at pH 4·0 and pH 8·6, and contained 99·9% of the theoretical phosphorus content. Inorganic orthophosphate was absent.

Glucose 6-phosphate and fructose 6-phosphate were obtained as a mixed sample of the barium salts from Dr C. Long.

Myosin and potato apyrase. Myosin was prepared from rabbit muscle by the method of Bailey (1942). However, in view of the laboriousness of the preparation and the rapid loss of activity on storage the use of myosin was

discontinued. Instead, a preparation of potato apyrase was made and tested by the method of Lee & Eiler (1951). The results obtained with this preparation were in good agreement with those of Lee & Eiler (1951, 1953).

Determination of phosphorus. Orthophosphate was determined by the method of Berenblum & Chain (1938) as modified by Long (1943). Total phosphorus was determined by digestion of the samples (0·5 ml.) with 0·5 ml. of the sulphuric–perchloric acid digestion mixture of Hanes & Isherwood (1949). After digestion the cooled solutions were adjusted to the phenolphthalein end-point with 20% (w/v) NaOH and diluted to 5–6 ml. with water. 10N-H_2SO_4 was then added to adjust the final acid concentration to N. The solutions were heated for 20–30 min. at 100° and cooled, and orthophosphate was estimated as above. To estimate total P in areas of paper after ionophoresis, the paper was first ashed (Wade & Morgan, 1954) before addition of the digestion mixture. By carrying out the whole procedure in Pyrex tubes (110 mm. × 20 mm.) the estimation of P was accomplished without transfer of solutions.

Paper ionophoresis was carried out either on Whatman no. 1 or no. 3MM paper. The latter paper was used for quantitative work. Before use all papers were washed first with N-HCl followed by 0·5% ethylenediaminetetraacetic acid (versene) in N-NH_3 soln., N-HCl, N acetic acid, and finally with glass-distilled water. A wash with glass-distilled water was given between each acid or alkaline wash. This treatment reduced the phosphorus content of the paper to 0·02–0·03 μg. of P/sq.cm.

The buffers used were either 0·05M Na acetate–acetic acid (pH 4·0), or 0·05M aminotrishydroxymethylmethane (tris) adjusted to pH 8·6 with HCl. The applied potential was 350–450v and a current density of 0·5–0·8 mA/cm. was maintained for 6–7 hr. The papers were removed, dried at 80° and developed either as described by Bandurski & Axelrod (1951) or by spraying with 0·25% (w/v) ammonium molybdate in N-H_2SO_4, and reheating at 80° until dry, respraying lightly and finally developing under an ultraviolet lamp (Osram or Mazda), from which the Woods glass filter had been removed. The phosphates appeared blue on a white background. With quantitative chromatograms from which the phosphates were to be eluted, guide strips of the pure phosphates were included in the run. These were later cut off and developed separately.

Extraction of phosphates from cerebral tissue and fractionation of the extract

Cerebral tissues (200–300 mg. wet wt.) were transferred to 3 ml. of ice-cold 10% (w/v) trichloroacetic acid (TCA) and were brought into a fine suspension by grinding in a glass tube with a fitting pestle. The suspension was centrifuged at 25 000 *g*, at −5° in the superspeed head of the M.S.E. Major refrigerated centrifuge, for 15 min. The residues were re-extracted with 2 ml. of 5% (w/v) TCA and the combined supernatants were adjusted to a definite pink phenolphthalein end-point with 5N and 0·1N-KOH. Then 1 ml. of 25% (w/v) barium acetate was added; the suspension was kept at 0° for 15 min. and was then centrifuged at 4500 *g* at 0° for 15 min. The residue was dissolved in 2 ml. of 0·1N-HCl and reprecipitated at pH 8·0 with 0·1 ml. of barium acetate solution. After 15 min. at 0° the residue was centrifuged and yielded the phosphates with insoluble barium salts.

The combined supernatants were precipitated with 5 vol. of 95% (w/v) ethanol, and sufficient 5N-KOH was added to produce a definite pink end-point to phenolphthalein. The solution, after 1–2 hr. at 0°, was centrifuged, yielding the phosphates with barium salts soluble in water but insoluble in ethanol.

Removal of barium from the residue

Phosphates with barium salts insoluble in water. This fraction was suspended in 3 ml. of water and shaken with 200–300 mg. of Zeo-Karb 315 (H^+) (90–120 mesh) (Permutit Co. Ltd., London) for 3–5 min. at 2°. The suspension was centrifuged and the residue washed twice with 2 ml. of water. The combined supernatants were transferred to a glass bulb tapering to a point, and a drop of conc. NH_3 soln. was added and the solution frozen at −20°. The water was evaporated under vacuum over P_2O_5 at 2°. The residue was taken up in 0·1 or 0·2 ml. of water, centrifuged to remove some impurities and the supernatants were measured on to paper.

Phosphates with barium salts soluble in water but precipitated by ethanol. This fraction was dissolved in 3 ml. of water at 0° and shaken with 200–300 mg. of Zeo-Karb 315 (NH_4^+) for 1 min. It was centrifuged promptly at 0°, the residue extracted twice with 2 ml. of ice-cold water and the supernatants were treated as described above. In view of the lability of creatine phosphate this operation was performed as rapidly as possible.

Tissues and salines

Slices of cerebral cortex of guinea pigs were cut as described previously by McIlwain (1951) and were incubated in conical flasks in a saline buffered with tris (Heald, 1954) for 30 min. For experiments in which concentrations of phosphates *in vivo* were to be determined, guinea pigs (100–200 g. wt.) were drowned in liquid oxygen and samples of whole brain were obtained as described by Kratzing & Narayanaswami (1953).

RESULTS

Ionophoresis of phosphates. In Table 1 are shown the R_P values of phosphates, most of which have been found to occur in extracts of brain (Stone, 1943; LePage, 1946), when subjected to ionophoresis at two different pH values. The figures quoted do not give an indication of the degree of overlap of two components moving close together. Thus ATP and inorganic phosphate (P_i) though apparently separated in acetate at pH 4·0 nevertheless suffered from a degree of overlap (cf. Wade & Morgan, 1954). At pH 8·6 ATP and ADP were not separable but were consistently separated from P_i. At this pH also, P_i, creatine phosphate, adenylic acid, diphosphopyridine nucleotide (DPN) and the hexose monophosphates were well separated. The hexose monophosphates were not separable from one another at this pH (cf. Neil & Walker, 1954). In experiments, not shown, at pH 7·4 in tris buffer, the following groups of phosphates were not separated: ATP, ADP and P_i; creatine phosphate and hexose monophosphates.

Precipitation of the phosphates with barium salts insoluble in water. In Table 2 are shown the amounts of P_i precipitated by 1 ml. of 25% (w/v) barium acetate in 15 min. at 0° from trichloroacetic acid solutions made alkaline with KOH. The volumes did not exceed 10 ml. The amounts of P_i and ATP used are such as are likely to occur in work with cerebral slices. In all experiments, the residues from the first precipitation were redissolved in 2·0 ml. of ice-cold 0·1N-HCl and reprecipitated by adding 0·1 ml. of 25% (w/v) barium acetate and making alkaline with KOH (Cori & Cori, 1931).

Although the recoveries appeared adequate (92–94%), experiments with $^{32}P_i$ present showed that appreciable amounts were not precipitated but remained in the supernatants; increasing the amount of carrier P_i fivefold reduced the amount of $^{32}P_i$ escaping precipitation only by half. The difference between the two types of estimation is attributed to the failure of the method of phosphate estimation to detect differences of the order of 0·2–0·5 μg.

Table 1. *Rate of movement of phosphates relative to the rate of movement of inorganic orthophosphate (R_P) during paper ionophoresis*

Applied potential: 400–450v. Current density: 0·5–0·8 mA/cm. Buffers, 0·05M. Paper, Whatman no. 3MM. Average length of run, 6 hr. Values are calculated from the relative distances of the centres of the phosphate spots.

Phosphate	R_P value: In acetate at pH 4·0	R_P value: In tris buffer at pH 8·6
Orthophosphate	1·0	1·0
Adenosine triphosphate	0·90	0·70
Adenosine diphosphate	0·75	0·70
α-Glycerophosphate	—	1·0
Fructose 1:6-diphosphate	1·0	1·0
Creatine phosphate	—	0·88
Glucose 6-phosphate / Fructose 6-phosphate	0·72	0·75
Adenylic acid	—	0·61
Diphosphopyridine nucleotide	—	0·31
Ethanolamine phosphate	0·61	0·67
Inosine triphosphate	0·90	0·70

Precipitation of ATP under similar conditions, at a quantity of 1·0 μmole/ml., resulted in 1·57 ± 0·2% (4) of the amount taken remaining in the supernatant. This quantity was considered to be sufficiently small to be neglected. Ennor & Stocken (1948) similarly found that the precipitation of ATP by barium was virtually quantitative. KOH was used for neutralization of the trichloroacetic acid solution since the use of NaOH with muscle extracts results in incomplete precipitation of P_i at pH 8·2 (Kosterlitz & Ritchie, 1943; Ennor & Stocken, 1948).

Precipitation of ATP and P_i together was found to be almost quantitative; thus with 0·55 μmole of P_i and 0·8 μmole of ATP added, recoveries were 0·556 ± 0·007 μmole of P_i and 0·82 ± 0·022 (5) μmole of ATP; and with 0·487 μmole of P_i and 0·354 μmole of ATP added, recoveries were 0·467 ± 0·029 (6) μmole of P_i and 0·330 ± 0·017 (6) μmoles of ATP.

Precipitation of the phosphates with barium salts soluble in water but precipitated by ethanol. The phosphates used to study the precipitation of this fraction were pure glucose 1-phosphate and creatine phosphate. In a final volume of 10–12 ml. there was little difference in the amounts of glucose 1-phosphate precipitated by 4 or 5 vol. of alkaline ethanol, but a marked difference when the final vol. was 50–60 ml. (Table 3).

The precipitation of creatine phosphate from solution was incomplete in the absence of a carrier phosphate, but was quantitative in the presence of glucose 1-phosphate. Thus, in a final vol. of 60 ml., the precipitation of barium creatine phosphate by 5 vol. of alkaline ethanol yielded these results: with carrier absent and 0·362 μmole of creatine phosphate present, recoveries were 0·168 μmole with a range of 0·115–0·213 μmole: with 0·815 μmole of glucose 1-phosphate present, recoveries were 0·393 ± 0·045 (5) μmole of creatine phosphate.

Removal of barium from the precipitates. The presence of traces of insoluble barium salts is known to cause marked streaking of chromatograms of phosphate esters (Hanes & Isherwood, 1949), and this occurred with paper ionophoresis at

Table 2. *Recovery of inorganic and radioactive orthophosphate when precipitated as the barium salts from 10 ml. of solution*

Conditions as described in Methods. Values are given ± S.E.M. Figures in parentheses indicate the number of determinations.

P_i added (μmole): With $^{32}P_i$	P_i added (μmole): Without $^{32}P_i$	P_i found in residue (μmole)	$^{32}P_i$ found (counts/min. as % of counts/min. taken): In residue	$^{32}P_i$ found (counts/min. as % of counts/min. taken): In supernatant
—	0·20	0·21 ± 0·003 (7)	—	—
—	0·23	0·21 ± 0·004 (4)	—	—
—	1·03	0·95 ± 0·004 (4)	—	—
0·49	—	—	88·2 ± 0·3 (8)	11·8 ± 0·5 (8)
2·65	—	—	94·6 ± 0·7 (6)	5·3 ± 0·7 (6)

pH 8·6. Exceptions to this were the calcium and barium salts of creatine phosphate and of glucose 1-phosphate which, being soluble in water, permit a movement of the cation towards the negative electrode while the phosphate moves towards the positive electrode. However, excess barium precipitated, presumably as carbonate, with the phosphates necessitated removal before ionophoresis. It was found simplest to remove barium by decomposition with a cation-exchange resin.

The recovery of ATP, P_i and creatine phosphate after precipitation as the barium salt and decomposition with the resin Zeo-Karb 315 is shown in Table 4. It will be seen that recoveries of ATP in the presence and absence of P_i was some 76%, while the recovery of P_i was 85–90%. Recoveries of 82% were obtained for creatine phosphate, some of which had decomposed during the treatment. In early experiments it was found that treatment of pure ATP (Preparation 3) for 30–40 min. at 0° with Zeo-Karb 315 led to the formation of a compound which moved slightly faster than ATP. This compound showed a sharp absorption band with a peak at 260 mμ. and had a ratio of acid labile P to total P of 7:10 (cf. Lund, Grylls & Harrison, 1954). The quantity of the material was reduced if the period of contact with the resin was reduced.

The solutions after treatment could be freeze-dried to yield residues which when taken up in water and subjected to ionophoresis showed well-defined spots without any streaking. The recoveries of ATP and of creatine phosphate when solutions were subjected to the whole procedure are about 80% (Table 5). This was considered satisfactory in view of the number of steps involved.

Table 3. *Recovery of glucose 1-phosphate by precipitation of the barium salt from alkaline ethanolic solution at* 0°

Values are given ± S.E.M. Figures in parentheses indicate the number of experiments.

Glucose 1-phosphate added (μmole)	Ethanol added to aq. soln. (vol.)	Final concn. of ethanol soln. (%)	Final vol. of solution (ml.)	Glucose 1-phosphate	
				Found (μmole)	Recovered (%)
0·25	4·0	76	10	0·19 ± 0·004 (3)	76
0·25	5·0	80	12	0·20 ± 0·004 (9)	80
0·49	4·0	76	10	0·42 ± 0·009 (3)	86
0·49	5·0	80	12	0·45 ± 0·013 (7)	92
0·49	4·0	76	50	0·35 ± 0·035 (6)	71
0·49	5·0	80	60	0·40 ± 0·027 (7)	82

Table 4. *Recovery of adenosine triphosphate, orthophosphate and of creatine phosphate after precipitation of the barium salts and decomposition with Zeo-Karb* 315

The ATP and P_i precipitate was decomposed with Zeo-Karb 315 (hydrogen-ion form), while the creatine phosphate (CrP) precipitate was decomposed with Zeo-Karb 315 (ammonium form). The latter phosphate was precipitated in the presence of 1·29 μmoles of hexose monophosphate. Values are given ± S.E.M. Figures in parentheses are the number of determinations.

Phosphate added (μmole)			Phosphate recovered (μmole)		
P_i	ATP	CrP	P_i	ATP	CrP
0	0·31	0	0	0·24 ± 0·016 (4)	0
0·48	0·31	0	0·44 ± 0·009 (5)	0·24 ± 0·014 (5)	0
0·48	0·63	0	0·42 ± 0·006 (3)	0·45 ± 0·01 (3)	0
0	0	0·63	0·029	0	0·52 ± 0·011 (6)

Table 5. *Recovery of adenosine triphosphate and creatine phosphate after precipitation as the barium salts, and decomposition with Zeo-Karb* 315, *and paper ionophoresis at* pH 8·6

Phosphate was determined as total phosphate after location on the paper strips. Values are given ± S.E.M. Figures in parentheses indicate the number of determinations.

Phosphate taken (μmole)		Phosphate found (μmole)		Phosphate found (as % of amount taken)	
ATP	CrP	ATP	CrP	ATP	CrP
0·52	0	0·44 ± 0·014 (5)	0	84 ± 2·7 (5)	0
0·78	0	0·61 ± 0·03 (6)	0	79 ± 4·1 (6)	0
0	0·63	0	0·52 ± 0·03 (6)	0	83 ± 5·5 (6)

Analysis of cerebral tissue

Tissues were denatured in ice-cold trichloroacetic acid, and were then centrifuged to remove debris. The efficiency of this process in two different centrifugal fields is shown in Table 6. In a high centrifugal field, removal of debris was complete and the solutions were quite clear. Very little additional phosphorus was extracted from the residue by a further two or three extractions. In contrast in a low centrifugal field, the supernatants are opaque and a second extraction yielded 50% as much phosphorus as the first extraction. This material was phospholipid (G. H. Sloane-Stanley, personal communication). It seems clear that with trichloroacetic acid extracts of cerebral tissues, the use of low centrifugal fields can lead to spuriously high values for the extractable phosphorus.

Qualitative examination of phosphates found when extracts were subjected to the above procedure showed spots corresponding to ATP and ADP, P_i, hexose monophosphate, creatine phosphate and a group labelled '*A*' containing both P_i which was not entirely precipitated by barium and probably any triose phosphates present. The phosphates soluble in ethanol were not examined further beyond ascertaining the quantity of phosphorus present. The area corresponding to ATP could have contained inosine triphosphate, since this was not separable from ATP. However, an eluate of the area showed a single sharp absorption peak at 260 mμ., the quotient E_{275}/E_{260} being 0·46, which compares with a value of 0·41 reported by Schmitz, Hurlbert & Potter (1954). In addition, fractionation of the phosphates having insoluble barium salts on a Dowex-1 column by the method of Utter, Kurahashi & Rose (1954) failed to reveal any material absorbing at 250 mμ. in eluates which showed such absorption when on authentic sample of inosine triphosphate was fractionated on the same column. Inosine triphosphate was considered to be absent.

The identity of the phosphate in the area corresponding to creatine phosphate was established by elution and subsequent determination of the creatine/phosphorus ratio. In two experiments this ratio was 1·0 and 0·95 respectively, from which it was concluded that the phosphate was creatine phosphate. No attempt was made to elute and identify the hexose monophosphate group.

Quantitative analysis of phosphates. The results of the separation and estimation of phosphate in cerebral tissue from guinea pigs are shown in Table 7 and are compared with similar data found for guinea-pig brain by other authors who had used different techniques. The values found are generally in good agreement with values obtained by other workers. A noticeable exception is the hexose monophosphate group, which will be discussed below. The group '*A*' forms too large a quantity to consist merely of P_i that escapes precipitation by barium, and probably contains triose phosphates.

The phosphate added to the paper was accounted for in the fractions estimated. Thus for the phosphates with insoluble barium salts $103 \pm 3{\cdot}8$% (4) of the phosphate added to the paper was accounted for as the sum of ATP and P_i, and for the phosphates soluble in water but precipitated by ethanol $96{\cdot}1 \pm 7{\cdot}5$% (6) was accounted for. Later experiments with $^{32}P_i$ confirmed these results and showed that no labelled phosphorylated intermediates could be detected as being absorbed by the resins (Heald, 1956).

In Table 8 is shown the degree of contamination of ATP, creatine phosphate and the hexose monophosphates with $^{32}P_i$ which was added to trichloroacetic acid extracts of cerebral tissue before fractionation. It is seen that the amount of contaminating ^{32}P appearing in the above compounds was well below that necessary to make the method of value in studies on the incorporation of ^{32}P into these fractions. Further, the results show that the use of an ion-exchange resin to remove barium did not promote any exchange between the ^{32}P in solution and phosphorus of the fractions. Not all the ^{32}P added was precipitated even by ethanol, for $1{\cdot}3 \pm 0{\cdot}3$% (4) of a count of 25 000 counts/min. added to a trichloroacetic acid extract was recovered in the fraction containing phosphates whose barium salts are soluble in water and ethanol. Whether this implies that inorganic phosphate is incompletely precipitated by ethanol and barium, or whether the samples of ^{32}P obtained contained other substances, is not known.

Table 6. *Effect of different centrifugal fields on the apparent amount of acid-soluble phosphorus extracted from cerebral tissue*

Slices of cerebral cortex were denatured in 10% (w/v) trichloroacetic acid, and the residues re-extracted with trichloroacetic acid. Total phosphorus was determined in each extract.

	P in extract (μmoles/g. wet wt. of tissue)			
	3500 *g*		25 000 *g*	
Extraction	(1)	(2)	(1)	(2)
1st	9·1	7·9	12·3	11·2
2nd	4·6	3·2	0·44	0·62
3rd	3·1	2·4	0·34	0·19
4th	2·3	2·7	—	—
5th	1·4	1·7	—	—
Total phosphorus in all extracts	20·5	17·9	13·08	12·01

Table 7. *Phosphates of guinea-pig brain* in vivo *and* in vitro

For details of analysis see Methods. Values marked with an asterisk were obtained from tissues respiring in a phosphate-containing saline. Values are quoted ± S.E.M. Figures in parentheses indicate the number of determinations. Fraction '*A*' is given in terms of μmole of P/g. wet wt. of tissue.

Tissue preparation	Phosphates (μmoles/g. wet wt. of tissue)							Method of estimation	Authors
	ATP and ADP	P_i	CrP	Hexose monophosphates	Adenylic acid	DPN	'*A*'		
Cerebral-cortex slices *in vitro*	0·80 ±0·03 (7)	2·15 ±0·21 (10)	1·12 ±0·14 (9)	0·72 ±0·10 (9)	0·59 ±0·11 (7)	0·17 ±0·04 (5)	0·91 ±0·09 (6)	Ionophoresis on paper	Present work
	—	1·52 ±0·14 (14)	1·35 ±0·08 (14)	—	—	—	—	Barium separation	Heald (1954)
	—	3·08* ±0·31 (15)	1·46 ±0·2 (15)	—	—	—	—	Calcium ethanol separation	McIlwain, Buchel & Cheshire (1951)
	1·01 ±0·07 (5)	—	1·12 ±0·04 (6)	0·23	—	—	—	Enzymic	Kratzing & Narayanaswami (1953)
	1·09 ±0·09 (6)	4·92* ±0·39 (6)	1·19 ±0·09 (4)	—	—	—	—	Calcium ethanol separation	Kratzing & Narayanaswami (1953)
Cerebral-cortex slices									
(*a*) Fresh slices	—	—	—	—	—	0·23	—	Manometric	Gore, Ibbott & McIlwain (1950)
(*b*) In glucose saline	—	—	—	—	—	0·20	—		
Whole brain frozen *in vivo*	3·5 ±0·1 (3)	3·2 ±0·4 (3)	2·68 ±0·12 (4)	1·34 ±0·21 (4)	1·02 ±0·08 (4)	—	—	Ionophoresis on paper	Present work
	—	3·8	3·3	—	—	—	—	Calcium ethanol separation	McIlwain *et al.* (1951)
	3·28	—	2·14	0·26	—	—	—	Enzymic	Kratzing & Narayanaswami (1953)
	3·68	3·58	2·66	—	—	—	—	Calcium ethanol separation	Kratzing & Narayanaswami (1953)

Table 8. *Degree of contamination of adenosine triphosphate, creatine phosphate and hexose monophosphate in tissue extracts when separated from radioactive orthophosphate by precipitation and ionophoresis on paper*

For details see Methods. CrP: creatine phosphate; HMP: hexose monophosphate.

	Counts/min. in area estimated				Counts/min. as % of $^{32}P_i$ added		
Determination	ATP	CrP	HMP	P_i	ATP	CrP	HMP
1	11·0	—	—	9 136	0·12	—	—
2	23·5	28·0	7·6	10 297	0·22	0·27	0·07
3	5·7	0·0	3·0	6 000	0·09	0·0	0·05

DISCUSSION

The method of analysis of phosphates described here, although based on an initial separation of the phosphates into groups by means of barium fractionation, overcomes most of the defects inherent in that procedure when applied to extracts containing radioactive phosphate in various forms. Thus the further separation of the fractions by means of paper ionophoresis yields compounds or groups of compounds which can either be estimated after acid digestion or be eluted and analysed by other methods.

Separation of phosphates other than creatine phosphate into groups by barium fractionation and subsequent paper-partition chromatography has been employed by Bandurski & Axelrod (1951) and by Ganguli (1953). Ionophoresis on paper at pH 3·2 has been used by Wade & Morgan (1955), though at this pH creatine phosphate is slowly dephosphorylated, resulting in 'streaking' between this ester and orthophosphate. A similar result attends the use of the Hanes & Isherwood (1949) solvents (Caldwell, 1953). In this respect ionophoresis at pH 8·6 appears to be advantageous.

Separation of the groups of phosphates containing adenosine di- and tri-phosphates and orthophosphate and hexose diphosphate is not complete, the resolution being into the adenyl phosphates, and orthophosphate plus hexose diphosphate. However, the use of an enzymic method for the determination of the terminal phosphate of adenosine triphosphate reduces possible errors of contamination by adenosine diphosphate. In cerebral extracts hexose diphosphate is reported as being absent or present in extremely low quantity, i.e. 0·1 μmole/g. wet wt. (LePage, 1946; Albaum, Tepperman & Bodansky, 1946), and does not constitute a grave error if included with orthophosphate.

The quantities of hexose phosphates found by the methods described above are markedly greater than those found by Kratzing & Narayanaswami (1953), whose value included any triose phosphates present in the extracts. Since their method of analysis must be presumed to estimate all the hexose monophosphate present (cf. Slater, 1953) the only obvious difference lies in the preparation of the extract. Thus Kratzing & Narayanaswami denatured the tissue in ice-cold perchloric acid and removed the perchlorate by conversion into the potassium salt and cooling in ice, when a large part of the potassium perchlorate crystallized. Possibly perchloric acid does not extract all the phosphates soluble in trichloroacetic acid, or the crystalline perchlorate adsorbs some phosphates such as hexose phosphates.

SUMMARY

1. A system of analysis of acid-soluble phosphates found in cerebral tissues, based on a paper-ionophoretic method, is described for use in the study of the metabolism of radioactive phosphorus.

2. Trichloroacetic acid extracts of tissue are first fractionated into groups by barium precipitation. The barium salts are then decomposed by a cation-exchange resin, and the resulting solutions are freeze-dried, before separation of the phosphates by ionophoresis.

3. The analysis of guinea-pig cerebral tissue by the method yielded results agreeing with values determined by other methods for some of the phosphates.

4. The separation and precipitation of the barium salts of several phosphates has been re-examined from the point of view of the small quantities of the phosphates normally encountered in the analysis of cerebral slices.

5. The centrifuging procedure employed can have a marked effect on the apparent total acid-soluble phosphorus extractable by trichloroacetic acid.

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Effects of Electrical Pulses on the Distribution of Radioactive Phosphate in Cerebral Tissues

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It is now well established that in nervous tissue, as in muscle, the application of fluctuating electrical potentials results in marked changes in the levels, both of creatine phosphate and of tissue inorganic phosphate (see McIlwain, 1952, for review). Thus in both tissues the level of creatine phosphate falls while that of inorganic phosphate rises. Information regarding the nature of this change in cerebral tissues has, however, been lacking, and the questions of whether the increased inorganic phosphate does indeed arise from creatine phosphate, and of the route through which the phosphate of creatine phosphate passes during breakdown, do not appear to have been investigated. The problem assumes a greater significance when it is realized that the tissues containing creatine phosphate are those which, owing to the suddenness of events taking place, require a large and immediate source of energy.

In muscle the energy used appears largely in the form of mechanical work, but in cerebral tissues the energy requirements appear to be for the maintenance or re-establishment of a steady state in the continuous ionic changes associated with the manifestation of electrical activity detectable, for example, by the electroencephalograph. Initially, therefore, it might not seem unreasonable to suppose that the pathway of creatine phosphate metabolism in brain may differ in some respects from that in muscle.

During an investigation of the rapid changes in slices of cerebral cortex of guinea pigs brought about by electrical pulses (Heald, 1954) it was found that the rate of breakdown of creatine phosphate appeared to be higher than that required to provide the 'energy-rich' phosphate involved in the metabolism of glucose. From this it was suggested that the 'energy-rich' phosphate released in response to pulses was not used solely in the phosphorylation of glucose. In an attempt to study this problem further, use has been made of radioactive phosphorus and the results obtained are described in this paper.

MATERIALS AND METHODS

Adenosine triphosphate, creatine phosphate and glucose 1-phosphate. These were samples described previously (Heald, 1956).