The Incorporation of Phosphate into Cerebral Phosphoprotein Promoted by Electrical Impulses

By P. J. HEALD
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

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It has been shown (Heald, 1956*b*) in cerebral slices that during the rapid breakdown of phosphocreatine, brought about by brief passage of electrical pulses (see Heald, 1954), there is an increased exchange of phosphate between the phosphates soluble in trichloroacetic acid and those in the insoluble residue. Thus in cerebral slices which had been allowed to metabolize radioactive inorganic phosphate for a brief period, electrical pulses applied for only 10 sec. caused a decrease in the specific radioactivity of both phosphocreatine and the γ-phosphate of adenosine triphosphate, and an increase in the specific radioactivity of the phosphorus of the tissue residue remaining after removal of the phosphates soluble in trichloroacetic acid. It was suggested that these changes could be resonably interpreted upon the basis of a cyclic transfer of phosphate between phosphocreatine, adenosine triphosphate and some unknown tissue phosphate or phosphates.

This paper describes experiments implicating a phosphoprotein fraction in such an exchange. An account of this work was presented to the Biochemical Society in November 1956 (Heald, 1957).

MATERIALS AND METHODS

Tissues and media. Slices of cerebral cortex from the guinea pig were cut and prepared as described by Heald, (1956*a*), with the media described in that paper. The slices were held and incubated in the rapid-transfer apparatus of Heald & McIlwain (1956) under the conditions described by Heald (1956*a*, *b*). Thus the slices were incubated in a glucose-containing medium buffered with 2-amino-2-hydroxymethylpropane-1:3-diol (tris) for 30 min. and then for precisely 3 min. in the presence of radioactive phosphate (carrier free). They were washed for 10 sec. in medium, and re-incubated for a further 2 min. in a fresh medium before fixing in ice-cold 10% (w/v) trichloroacetic acid.

When electrical pulses were applied they were switched on 10 sec. before the tissue was fixed. In such experiments slices from one cerebral hemisphere formed the controls for slices from the other cerebral hemisphere.

Electrical-condenser pulses. These were applied from the apparatus of Ayres & McIlwain (1953) at a frequency of 50 cyc./sec., a peak potential of 18 v and a duration of 0·4 msec. being used.

Radioactive phosphorus was obtained as carrier-free orthophosphoric acid, and was treated as described by Heald (1956*a*) before use.

Treatment of the tissue after incubation

After incubation, the slices (about 500–800 mg. total wet wt.) were fixed in 10% (w/v) trichloroacetic acid (5 ml./100 mg. of slices) and ground to a fine suspension in a small glass tube with a fitting pestle. The suspension was then centrifuged at 20 000 *g* for 10 min. in the superspeed attachment of the MSE Major centrifuge at −5°. The residues were further fractionated by a method essentially similar to that of Strickland (1952). Residues were washed five times with 5% (w/v) trichloroacetic acid to remove the bulk of the adsorbed radioactive phosphate, and then extracted twice, by trituration for a few minutes with 5 or 6 vol. of $CHCl_3$–methanol (2:1, v/v) at 0° to remove most of the phospholipids. Samples of this extract were taken for the determination of total phosphorus and radioactivity. The residue from the phospholipid extraction was treated for 5 min. at room temperature with 1 ml. of 10 M-urea, and then with 2 ml. of a saturated solution of NaCl which had been further saturated at boiling point with $(NH_4)_2SO_4$ and cooled. The mixture was heated at 100° for 1 min., and cooled and centrifuged, the supernatants being retained. The residue was re-extracted three times at 100° for 1 min., with the saturated salt solution containing 15% (w/v) urea. The supernatants were combined and 1 ml. of saturated $CuSO_4$ solution was added to precipitate the nucleic acids. After standing overnight at 2°, the precipitate was centrifuged and digested for the determination of total phosphorus and radioactivity. This procedure for extraction of the nucleic acids is essentially that of Hammarsten (1947) and is stated (cf. Strickland, 1952) to remove almost all the nucleic acids from a tissue.

After extraction of the nucleic acids, the tissue residue was digested with 5 ml. of N-KOH at 37·5° for 18 hr., when almost all dissolved. To the solution 0·2 vol. of 6 N-HCl and 0·5 vol. of 10% (w/v) trichloroacetic acid were added and the precipitate was centrifuged and washed in 2 N-HCl. This precipitate was digested for the determination of total phosphorus and radioactivity and constituted the 'acid precipitable' fraction.

A sample of the acid-soluble supernatant was digested for the determination of total phosphorus and a further sample used for the determination of inorganic phosphorus, presumed to arise from phosphoprotein (Logan, Mannel & Rossiter, 1952). The difference between these two determinations gave a value for the residual phosphorus.

Analytical methods

Inorganic phosphate. This was estimated by the method of Berenblum & Chain (1938) as modified by Long (1943).

Total phosphorus. This was estimated by digestion of appropriate samples with the sulphuric acid–perchloric acid mixture of Hanes & Isherwood (1949). After hydrolysis of any pyrophosphate present (see Heald, 1956*a*) orthophosphate was estimated as described above.

Units. The specific radioactivity of a phosphate is defined as counts/min./μg. of phosphorus. The relative specific radioactivity is defined as counts/min./μg. relative to the specific radioactivity of the original incubation medium. This latter was not maintained within fairly narrow limits as previously described (Heald, 1956*b*), but in each experiment the average was taken of the specific radioactivities of the media used for control and experimental tissues. In any one experiment the difference in specific radioactivity between the control and experimental media was usually so small as to be almost negligible.

RESULTS

Removal of contaminating radioactive phosphate. When radioactive phosphate of high specific radioactivity is used, the problem of contamination of tissue residues with traces of adsorbed phosphate can assume considerable proportions (Davidson, Frazer & Hutchison, 1951), and it was considered desirable to try to remove as much contaminating material as possible before attempting to fractionate the tissue residues. Strickland (1952, 1954), using cat cerebral tissue both *in vivo* and *in vitro*, found that radioactive inorganic phosphate could be removed from the tissue residues by seven washings with trichloroacetic acid containing inactive carrier phosphate. Similar results were reported by Ennor & Rosenberg (1954) with liver. These results are in contrast with those of Davidson *et al.* (1951) who

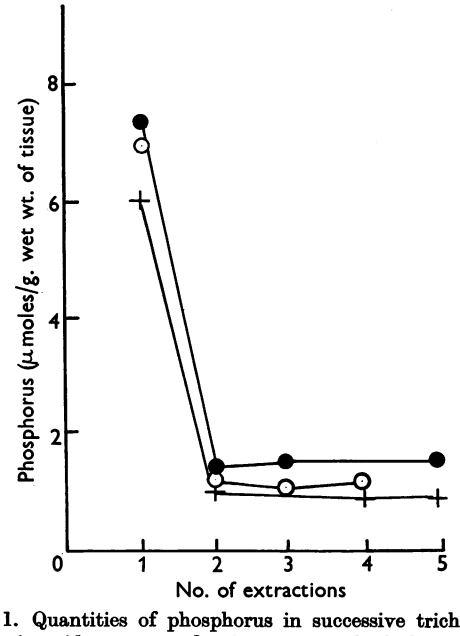

Fig. 1. Quantities of phosphorus in successive trichloroacetic acid extracts of guinea-pig cerebral slices. The different symbols refer to individual experiments.

Table 1. *Removal of added radioactive phosphate from tissue treated with trichloroacetic acid by washing with* 10% (*w/v*) *trichloroacetic acid*

Generally, 300–400 mg. of tissue was precipitated with trichloroacetic acid (10% w/v) containing carrier-free ^{32}P, and the residue re-extracted with 3 ml. portions of trichloroacetic acid. All operations were at 0°.

	Counts/min.					
	Extract					
Expt. no.	1	2	3	4	5	Tissue residue
1	10 216	597	55	0·3	6·0	22·0
2	9 987	607	40	3·0	0·0	31·0
3	6 965	405	35	15·0	4·0	—
4	7 372	437	53	54	0·0	—

Table 2. *Absence of radioactivity in various cerebral tissue fractions after washing the tissue residue with trichloroacetic acid*

Conditions were as described in Methods and Table 1. The expts. correspond to those in Table 1.

	Fraction counted				
Expt. no.	Phospholipids	Nucleic acids	Acid-precipitable	Phospho-protein	Residual
1	19·0	2·0	1·0	0	1·0
2	32·0	0·0	0·0	0	0·0
3	—	—	0	0	0·0
4	—	—	0	0	0·0

were unable to remove traces of radioactive phosphate from tissue residues even after fifty extractions with trichloroacetic acid.

It was found that guinea-pig cerebral slices, when precipitated with trichloroacetic acid containing radioactive phosphate, could be washed free of radioactivity by five consecutive washings with fresh precipitant (Table 1). The counts remaining in the fourth and fifth washes were so low as to be of little significance. Determination of the specific radioactivity of the total phosphate extracted by such successive extraction showed that this progressively decreased, though here the low values in the fourth and fifth extracts are subject to error because of the low counts present (see Table 1). Since appreciable quantities of phosphorus were present even in the last few extracts (Fig. 1), these results were taken to mean that continued trichloroacetic acid extraction causes a slow breakdown of some phosphorus-containing material. Consequently the number of extractions was limited to five. Fractionation of the tissue residue to yield the phospholipid, nucleic acid and phosphoprotein fractions showed that here also (Table 2), as expected from the results in Table 1, little if any radioactivity could be detected in these fractions (cf. Ennor & Rosenberg, 1954). If anything such contamination as did occur was in the phospholipid fraction.

The quantities of radioactive phosphate employed here were those which had previously been found in trichloroacetic acid extracts of some 300–400 mg. of tissue after incubating as described above in the presence of 5–6 μc of ^{32}P for each 100 mg. of slices. Consequently, on the basis of these results it was considered that, provided the amounts of radioactive phosphate added to the medium were kept within these limits, physical contamination of any tissue fraction by inorganic phosphate from the medium was not likely to cause appreciable error.

Metabolic incorporation of radioactive phosphate in the presence and absence of electrical pulses

The incorporation of phosphate into the phospholipids and non-phospholipids of cerebral slices was first studied to see which major fraction increased in radioactivity on passage of pulses. The results of nine such experiments presented in Table 3 showed quite clearly that the change taking place occurred in the non-phospholipid fraction. It is to be noted, however, that the phospholipid fraction contains some ten times more phosphorus than does the non-phospholipid fraction, namely 60 and 6–7 μmoles of phosphorus/g. wet wt. of tissue respectively. On this basis it can be calculated that the total amount of radioactivity incorporated into the phospholipid fraction was between 30 and 50 % of the total incorporated into the residue.

Further fractionation of the non-phospholipid fraction revealed that the increase in activity occurred solely in the phosphorus of the 'phosphoprotein' fraction (Table 4), at a high degree of significance. Incorporation into the nucleic acid fraction was small and variable, as was incorporation into the acid-precipitable fraction and the residual fraction. This latter fraction showed greater variability than any other, and probably reflects the errors in the determination of total phosphorus and specific radioactivity of this fraction combined with the errors in determination of the phosphoprotein fraction. These latter, however, should be small, since the precision of the method of inorganic phosphate determination used is high.

Table 3. *Distribution of radioactive phosphate between tissue phospholipids and residual phosphates in the presence and absence of electrical pulses*

Tissues were precipitated with trichloroacetic acid (10%, w/v) and the residue was extracted with $CHCl_3$–methanol (2:1, v/v) as described in Methods. Pulses were applied to one sample of tissue in each experiment, peak voltage 18v, duration 0·4–0·5 msec., frequency 50 cyc./sec. Different amounts of carrier-free radioactive phosphate were used for each experiment.

	Specific radioactivity of phosphate					
	$CHCl_3$–methanol extract			Residue		
Expt. no.	With pulses (1)	Without pulses (2)	(1) – (2)	With pulses (3)	Without pulses (4)	(3) – (4)
1	0·25	0·55	−0·30	15·0	11·0	+4·0
2	1·13	1·60	−0·47	11·7	13·7	−2·0
3	2·6	1·20	+1·40	51·0	33·8	+17·2
4	0·37	0·30	+0·07	7·5	5·9	+1·6
5	1·02	1·00	+0·02	14·5	12·8	+1·7
6	0·75	0·52	+0·23	13·3	7·55	+5·75
7	0·32	0·22	+0·10	8·05	8·20	−0·15
8	0·84	1·13	−0·29	14·50	10·30	+4·20
9	0·55	0·36	+0·19	4·90	4·10	+0·8

Table 4. *Effect of electrical pulses upon the distribution of radioactive phosphorus in various cerebral fractions*

Tissues were fractionated as described in Methods. Pulses, condenser pulses, 50 cyc./sec., peak potential 18 v, duration 0·4 msec. Values given are relative specific radioactivities.

	Fraction											
	Nucleic acids			Acid-precipitable			Phosphoprotein			Residual		
Expt. no.	With pulses	Without pulses	Diff.	With pulses	Without pulses	Diff.	With pulses	Without pulses	Diff.	With pulses	Without pulses	Diff.
1	0·93	0·62	+0·31	3·8	2·7	+1·1	12·5	17·3	− 4·8	4·0	4·2	−0·2
2	0·90	1·31	−0·41	10·5	13·6	−3·1	36·0	37·0	− 1·0	4·8	5·4	−0·6
3	2·02	2·80	−0·78	17·5	8·4	+7·1	77·0	34·6	+32·4	6·9	—	—
4	0·79	0·40	+0·39	0·84	1·23	−0·39	48·0	32·0	+16·0	—	—	—
5	3·22	1·03	+2·19	4·75	2·5	+2·19	72·0	50·0	+22·0	—	—	—
6	—	—	—	6·60	4·90	+1·70	96·0	73·0	+23·0	6·50	4·95	+1·55
7	9·5	10·0	−0·5	2·9	—	—	74·5	59·5	+20·0	3·76	10·0	−6·24
8	3·74	6·6	−2·96	6·95	4·92	+2·03	33·8	23·2	+10·6	13·6	16·7	−3·1
Significance of the differences			P=0·5–0·7			P=0·2–0·1			P=0·01–0·001			P=0·3–0·2

Table 5. *Distribution of phosphorus and radioactivity in various fractions of cerebral tissues of guinea pig*

Guinea-pig cerebral slices were incubated in a medium containing glucose and radioactive phosphate before fractionation. For details see Methods. Values are given ± S.E.M. Figures in parentheses indicate the number of determinations.

Phosphate fraction estimated	Phosphorus (μmoles/g. wet wt. of tissue)	Average specific radioactivity	Radioactivity in the various fractions (% of total)	Phosphorus (μmoles/g. wet wt. in cat cerebral fractions) (Strickland, 1952)
Phospholipid	58·3 ±1·90 (6)	0·76	35·6	—
Nucleic acid	1·45±0·1 (13)	4·5	5·2	4·19
Acid-insoluble phosphorus	1·23±0·12 (12)	6·4	6·5	0·3
Phosphoprotein fraction	1·16±0·1 (12)	40·8	38·0	0·9
Residual phosphorus	3·11±0·39 (10)	6·0	14·7	6·30

In Table 5 are shown the quantities of phosphorus present in the individual fractions, together with the percentage distribution of radioactivity between these fractions. These latter values were calculated from the average specific radioactivity of each fraction determined in experiments in which slices were not subject to pulses. Also presented for comparison are the phosphate levels in similar fractions of cat cerebral slices calculated from the data of Strickland (1952). The most outstanding point is that the phospholipid and phosphoprotein fractions between them account for 73 % of the total radioactivity incorporated, very little appearing elsewhere.

DISCUSSION

It has been found that in cerebral tissue subjected to electrical pulses in the presence of radioactive phosphate, there is a marked increase, averaging 60 %, in the radioactivity of the phosphorus considered to be derived from phosphoprotein. This change took place within 10 sec. of applying pulses and, assuming that all the phosphorus of this fraction is exchanged at least once within this period, occurs at a minimum rate of 400 μmoles/g. wet wt. of tissue/hr. The levels of phosphorus in this fraction did not change significantly during the passage of pulses. Thus the levels ± S.E.M. were: with pulses, 1·17 ± 0·1 μmoles of phosphorus/g. wet wt.; without pulses, 1·16 ± 0·1 μmoles of phosphorus/g. wet wt. These values show that the increase in radioactivity in response to pulses represents a metabolic exchange, in keeping with the suggested role of such a phosphate in a cyclic-transfer reaction. Since it has been shown (Heald, 1954) that phosphocreatine, also proposed as part of the transfer system, is metabolized at a rate of 1200–1400 μmoles/g. wet wt./hr., in response to pulses, the rate of 400 μmoles of phosphorus/g./hr. quoted above for the phosphoprotein fraction would need to be increased by a factor of 3 or 4 if this fraction is indeed a major component of the system.

Assessment of this possibility is rendered difficult since analysis of cerebral tissues for phosphates other than those extracted by acid denaturants has so far been confined to separation into ill-defined groups (see Heald, in preparation) and information regarding the nature of cerebral phosphoprotein is almost totally lacking. Indeed, apart from McGregor (1916), who isolated from brain a protein fraction

containing non-dialysable phosphorus, the majority of workers have analysed cerebral tissues for phosphoprotein by determination of the inorganic phosphate liberated from the tissue on alkaline digestion. In view of the complexity of cerebral acid-insoluble phosphates (see Sloane-Stanley, 1952; Rossiter, 1955), it is not unlikely that there are present in brain several compounds of high molecular weight, capable of yielding inorganic phosphate upon mild alkaline hydrolysis. Recently the view has been expressed (Engelhardt & Lissovskaya, 1953; Findlay, Strickland & Rossiter, 1954) that the phosphorus of cerebral phosphoprotein is in fact the phosphorus of enzyme–substrate complexes, though as yet no evidence for this has been put forward.

In spite of these limitations, it seems reasonable to suppose that analytical figures derived from a standard procedure should be capable of comparison, and on this basis it appears permissible to compare the metabolic activity of the phosphoprotein fraction found in this work with the results of other workers. It seems well established (Strickland, 1952, 1954; Johnson & Albert, 1953; Engelhardt & Lissovskaya, 1953; Lissovskaya, 1954) that the incorporation into cerebral phosphoprotein either *in vivo* or *in vitro* proceeds at a rate greater than that found for any other group of acid-insoluble tissue phosphates. This incorporation is dependent upon metabolically derived energy. Since these experiments were carried out over periods of a few hours, rates of incorporation are not comparable with those found above. However, it is clear that the phosphorus of the phosphoprotein fraction must turn over many times more rapidly than that of other groups of phosphates. It is of interest that Vladimirov (1953), in a review article, stated that *in vivo* the rate of turnover of cerebral phosphoprotein is at least equal to the rate of turnover of adenosine triphosphate and phosphocreatine, and was increased when the animal was convulsed either by electric shock or by camphor. In this respect Vladimirov's results compare with those described here *in vitro* and would imply that cerebral phosphoprotein is involved in some major metabolic role as yet not understood.

SUMMARY

1. It has been shown that application of electrical pulses to guinea-pig cerebral slices metabolizing radioactive phosphate results in an increase in activity of the phosphoprotein phosphorus. No other group of tissue components insoluble in trichloroacetic acid showed such a change.

2. The change occurs within 10 sec. and proceeds at a minimal rate of 400 μmoles/g. wet wt. of tissue/hr.

3. The results are discussed in relation to cerebral phosphoprotein metabolism.

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