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Phosphorylserine and Cerebral Phosphoprotein

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It has been shown previously that the phosphoprotein fraction of brain is implicated in the rapid changes in energy-rich phosphates which occur when electrical pulses are passed through cerebral slices *in vitro* (Heald, 1957*a*). This result was obtained when the cerebral tissues were initially fixed in trichloroacetic acid and the phosphoprotein fraction was determined by the method of Strickland (1952). Further work, in which brain slices were first fixed in a lipid extractant and later extracted with dilute alkali, gave no clear indication of the presence of such a phosphoprotein fraction in the tissue (Heald, 1957*b*). It was suggested that possibly the phosphoprotein, as originally measured, was a stable acid-degradation product of a more labile phosphate which was split to yield inorganic phosphate under alkaline conditions. To pursue this work it seemed necessary to try to characterize the phosphoprotein by means of a specific component and also to try to resolve the discrepancy mentioned above.

This paper describes experiments showing that the phosphoprotein fraction, when obtained from cerebral tissues which had first metabolized radioactive phosphorus and were subsequently fixed in trichloroacetic acid, contains radioactive phosphorylserine. When this work was almost completed the author became aware of a paper of Vladimirov, Ivanova & Pravdina (1956) also describing the isolation of phosphorylserine from brain. Identification of the product by these authors does not appear to have been entirely unequivocal.

MATERIALS AND METHODS

Tissues and media. Slices of guinea-pig cerebral cortex were incubated with radioactive phosphate in media containing glucose and buffered with 2-amino-2-hydroxymethylpropane-1:3-diol (Heald, 1956*b*). Generally six slices of 100–150 mg. wet wt. were obtained from each cortex. For short-term experiments, in which electrical pulses were applied for brief periods, the apparatus and techniques used were those described previously (Heald, 1956*b*). In such experiments, since the radioactivity of the phosphorylserine finally isolated was low, slices from two or three animals were pooled for each experiment. In all cases slices from one cerebral hemisphere served as controls for slices from the other hemisphere of any one animal.

Longer experiments, in which slices were incubated with radioactive phosphate for up to 90 min., were carried out in conventional manometric apparatus. All incubations were at 37·5° in an atmosphere of O_2.

Electrical pulses. These were condenser pulses supplied by the apparatus of Ayres & McIlwain (1953) at 50 cyc./sec. and were of 15v peak potential and a duration of 0·4–0·5 m-sec.

Radioactive phosphate. This was obtained as carrier-free orthophosphoric acid and was treated as described by Ennor & Rosenberg (1954) before use.

Preparation of the phosphoprotein fraction. After incubation, tissues were fixed either in 10% (w/v) trichloroacetic acid or in $CHCl_3$–methanol (2:1, v/v). From tissues

fixed in trichloroacetic acid, the phosphoprotein fraction was prepared by methods described previously (Heald, 1957*a*). With tissues fixed in $CHCl_3$–methanol, the extraction procedure described by Heald (1957*b*) was carried out up to the end of the washing with $(NH_4)_2SO_4$. At this point the tissue residues were either treated with ice-cold 10% (w/v) trichloroacetic acid directly or were extracted with aq. 0·33 M-NH_3 soln. before treatment with trichloroacetic acid. The residues were extracted to remove nucleic acid and to yield the phosphoprotein fraction. All residues were hydrolysed for 10 hr. at 100° in 2 N-HCl in the presence of phosphorylserine, added in quantities varying from 100 to 300 μg. of P, to act as an inactive carrier for radioactive phosphorylserine released on hydrolysis.

Separation of phosphorylserine from the hydrolysates. Phosphorylserine was separated from the acid hydrolysates by two methods. In the first method, which was a modification of that of Kennedy & Smith (1954), the hydrolysates were made alkaline to phenolphthalein with ammonia and diluted to reduce the concentration of Cl^- ion to below 0·01 M. The diluted hydrolysates were then passed through a column (10 cm. × 1 cm.^{2}) of Dowex-1 chloride (200–400 mesh; 4 or 8% cross-linked). The column was washed with water and the radioactive phosphates were eluted with 0·01 N-HCl. Both inorganic phosphate and phosphorylserine were eluted together. The residue obtained on freeze-drying the eluate was chromatographed on paper with the acetate–ethanol solvent of Kennedy & Smith (1954). Phosphorylserine was well separated from inorganic phosphate, phosphorylcholine and phosphoryltyrosine in this solvent. It was not separated from phosphorylthreonine or phosphorylhydroxyproline. After detecting phosphorylserine either with ninhydrin or the molybdate reagent of Hanes & Isherwood (1949) the spots were cut out and digested for the determination of phosphorus and radioactivity as described previously (Heald, 1956*a*). Paper chromatography was necessary since, in contrast with the results of Kennedy & Smith (1954), it was not found possible to remove all the radioactive inorganic phosphorus from the acid hydrolysates by magnesia mixture before ion-exchange chromatography.

In the second method, of which most use was made and which was essentially that of Schaffer, May & Summerson (1953), the acid hydrolysates were freeze-dried in the presence of NaOH pellets and the residues, dissolved in 2 ml. of 0·05 N-HCl, were chromatographed on a column (20 cm. × 3 cm.^{2}) of Dowex-50 (12% cross-linked) (a gift from Dr J. B. Jepson), with 0·05 N-HCl as the eluent. The separation of inorganic phosphate and phosphorylserine was extremely good. On this column inorganic phosphate appeared in fractions 4–10, phosphorylserine in fractions 25–35, and phosphorylthreonine in fractions 38–48. Reproducibility was good and the phosphorylserine eluted appeared to be almost entirely free from phosphorylthreonine. The tubes containing the phosphorylserine peak were combined and concentrated before samples were taken for the determination of the total phosphorus and radioactivity.

Phosphorylated amino acids. Phosphorylserine, phosphorylthreonine, phosphoryltyrosine, phosphorylhydroxyproline and phosphorylcholine were gifts from Dr G. R. Webster. Phosphorylserine, prepared from casein in collaboration with Dr O. E. Pratt, was also obtained by a method modified from that of Ågren, de Verdier & Glomset (1951).

Casein (750 g.) was extracted with hot ethanol and acetone and the residue was hydrolysed for 10 hr. under reflux with 3 l. of 2 N-HCl. The solution was cooled and made alkaline to phenolphthalein with conc. aq. NH_3 soln. Barium acetate (25 g. in 100 ml. of water) was added and after standing for 1 hr. the solution was filtered and the filtrate added to the washings obtained below. The residue was taken up in 500 ml. of water, the suspension was dissolved by addition of acetic acid, and barium phosphate was precipitated by making the solution alkaline with ammonia. The suspension was filtered and the residue was washed with water. The combined filtrates from all stages together with the washings amounted to 4 l. An equal volume of ethanol was added and after standing overnight the precipitate was filtered off. The residue was dissolved in 500 ml. of water, brought to pH 4·6 with acetic acid and filtered; the filtrate was treated with 50 ml. of saturated lead acetate. The precipitate was decomposed with H_2S and, after removing the H_2S in a stream of O_2, the lead salts were again precipitated. This was repeated four times. The final solution still contained much inorganic phosphate and was treated at pH 8·2 with barium acetate. After filtering, the barium salt of phosphorylserine was precipitated with 5 vol. of ethanol, yielding 0·5 g. The sample contained 2% of inorganic phosphorus and ran as a single phosphorus-containing ninhydrin-positive spot, not separable from synthetic phosphorylserine, in acetate–ethanol. On Dowex-50 the major phosphorus-containing peak coincided with that yielded by synthetic phosphorylserine on the same column.

Analytical methods. The methods used for the determination of phosphorus and radioactivity have been described previously (Heald, 1956*b*) and were used without modification.

Units. The specific radioactivity is defined as the counts/min./μg. of P. The relative specific radioactivity is defined as counts/min./μg. of P compared with the specific radioactivity of the inorganic phosphorus in the incubation medium. Methods and procedures for this have already been described (Heald, 1957*a*).

RESULTS

Identification of phosphorylserine as a component of cerebral phosphoprotein in tissues fixed in trichloroacetic acid

Since the amount of cerebral phosphoprotein is rather low (0·6–1·0 μmole of P/g. wet wt. of cerebral tissue) phosphorylserine in acid hydrolysates was identified by co-chromatography with inactive phosphorylserine added as a carrier. Tissues were incubated for 90 min. with 10–12 μc of radioactive phosphate/slice, fixed in trichloroacetic acid, and the phosphoprotein fraction was isolated and hydrolysed. When such a hydrolysate was chromatographed upon Dowex-50 two peaks were obtained (Fig. 1). The first peak corresponded to the position occupied by inorganic phosphate and the second corresponded to that occupied by phosphorylserine when inactive samples of these two phosphates were chromatographed upon the same

column. No other peaks were eluted by HCl in concentrations up to 6N. The second peak corresponded to 10–15% of the total counts in the esterified phosphorus present in the hydrolysate.

In Fig. 2 are shown the distributions of phosphorus and radioactivity in the individual fractions of the second peak of Fig. 1. Both followed the same curve, indicating that the peak consisted of a single entity. In another experiment the specific radioactivity of the total phosphorus in such a peak was 22·4. A sample of the pooled fractions composing the peak was freeze-dried and chromatographed upon paper in the acetate–ethanol solvent of Kennedy & Smith (1954). A single ninhydrin-positive spot containing radioactive phosphorus was detected running at the same rate as a specimen of synthetic phosphorylserine. No other component was detected either by ninhydrin or by measurements of radioactivity in other areas of the paper. Determination of the specific radioactivity of the spot yielded a value of 21·8, indicating no real change from the specific radioactivity of the original peak. When rechromatographed upon the same Dowex-50 column the second peak was re-eluted at the same point, no other components being present. It seemed clear that phosphorylserine was the only component of the second peak shown in Fig. 1. In experiments in which the hydrolysates had first been chromatographed upon Dowex-1 chloride and the radioactive peak rechromatographed upon paper, no spot containing both radioactive phosphorus and ninhydrin-positive material other than that corresponding to phosphorylserine could be detected.

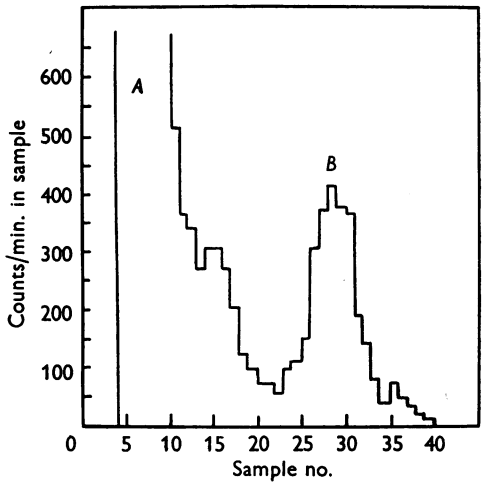

Fig. 1. Fractionation of an acid hydrolysate of a cerebral phosphoprotein fraction upon Dowex-50 (×12), (20 cm. × 3 cm.²). Eluent, 0·05N-HCl; fraction volume, 2·5 ml. Peak *A*, inorganic phosphate; peak *B*, phosphorylserine. Counts in peak *A* not recorded in the figure were: 2059, 27 798, 59 931, 31 892, 5264 and 1062 respectively.

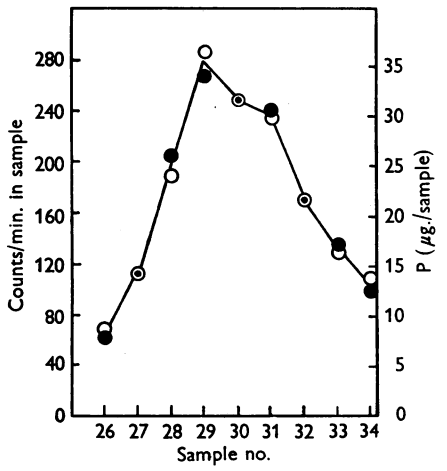

Fig. 2. Diagram showing the equivalence of total phosphorus (●) and radioactivity (○) in the individual fractions of peak *B* in Fig. 1.

Effect of electrical pulses upon the incorporation of phosphate into phosphorylserine

It has been shown previously that the rate of incorporation of radioactive phosphate into cerebral phosphoprotein is increased by the brief passage of electrical pulses through the cerebral slices (Heald, 1957*a*). Since phosphorylserine had been found to be a component of the phosphoprotein fraction it was of interest to see whether its specific radioactivity was also increased by passage of impulses under similar conditions. In these experiments identical amounts of tissue were used for the control and experimental slices and identical quantities of carrier phosphorylserine were added to the phosphoprotein residues before hydrolysis.

Table 1. *Effect of electrical pulses upon the specific radioactivity of phosphorylserine isolated from slices of guinea-pig cerebral cortex*

For experimental details see Methods. Pulses were condenser pulses, 15 v peak potential, 0·4 m-sec. duration. They were applied for 10 sec. Values are the specific radioactivities in counts/min./μg. of P.

Expt. no.	Specific radioactivity of phosphorylserine isolated: Slices with pulses	Slices without pulses	Increase with pulses
1	3·9	2·48	+1·42
2	6·51	5·75	+0·76
3	1·33	1·03	+0·31
4	3·85	2·80	+1·05

The results of applying pulses for 10 sec. to cerebral tissues which had been allowed to metabolize radioactive phosphate for 3 min. are shown in Table 1. In all cases pulses induced a marked increase in the specific radioactivity of the phosphorylserine.

Phosphorylserine from tissues fixed in chloroform–methanol

Since phosphorylserine could clearly be used as an indication of the presence of the phosphoprotein fraction being investigated, it was sought in residues from tissues which had first been degraded in chloroform–methanol and then treated with ammonia as described above. After such treatment, tissues were washed with trichloroacetic acid and the phosphoprotein fraction was isolated and hydrolysed. When fractionated upon Dowex-50, the hydrolysates of such experiments were found to contain a peak corresponding to the position occupied by phosphorylserine. In any one experiment the counts in this peak were roughly equal to the counts in a similar fraction derived from control tissues which had been initially fixed in trichloroacetic acid. Analysis of the peaks showed that the phosphorus content of each fraction when plotted against the counts/min. followed a curve similar to that shown in Fig. 1. Rechromatography of such a peak again yielded a single fraction, which was eluted from the column at the same point as the original fraction. When freeze-dried and chromatographed upon paper in acetate–ethanol, a single ninhydrin-positive phosphate-containing spot was detected. No other radioactive component was found on the paper. The presence of phosphorylserine in the hydrolysates was not due to combination of radioactive inorganic phosphate with a serine residue during hydrolysis, for when a non-radioactive phosphoprotein fraction was hydrolysed with added radioactive phosphorus only inorganic phosphate was detected upon chromatography of the freeze-dried hydrolysate upon Dowex-50.

DISCUSSION

The main features of the work described here are, first, the demonstration of phosphorylserine as the sole radioactive phosphorylated amino acid present in hydrolysates of radioactive cerebral phosphoprotein; and, secondly, that the phosphorylserine increased in specific radioactivity when derived from tissues metabolizing radioactive phosphate and subjected to electrical pulses for a brief period. Phosphorylserine is well known to be a component of many naturally occurring phosphoproteins (see review by Perlmann, 1955) and has been shown to be the sole phosphorylated amino acid which can be isolated from phosphoprotein fractions of liver or ascites cells (Kennedy & Smith, 1954) after these tissues had metabolized radioactive phosphate. The present results now add the phosphoprotein of brain to this list.

Although no major component other than phosphorylserine could be detected, it is to be noted that only 10–15% of the total combined radioactive phosphorus of the acid hydrolysates could be accounted for as the phosphorylated amino acid. This is a somewhat lower value than that of 30% which has been found in metabolic studies with the other phosphoproteins mentioned above. Possibly the differences here may lie in the nature of the phosphoprotein fraction which, in brain, contains relatively large amounts of phosphorus in the form of phosphoinositides and phosphatidopeptides.

The demonstration that the phosphorylserine isolated increases in specific radioactivity in response to electrical pulses clearly indicates that it is derived from part of the phosphoprotein molecule which has previously been shown to take part in phosphate-transfer reactions in the intact tissue slices (Heald, 1956*b*, 1957*a*, *b*). Evidence which has been presented previously (Heald, 1956*b*, 1957*a*) indicates strongly that in brain this process is of a cyclical nature. In this respect phosphorylserine appears to constitute an anomaly, for such reactions require that the components taking part can transfer energy in the form of phosphate bonds and are thus presumably labile in acid conditions, whereas phosphorylserine itself is remarkably stable to acid hydrolysis (Plimmer, 1941). Whether phosphorylserine is merely the end product of a hydrolysed protein pyrophosphate (Burnett & Kennedy, 1954; Perlmann, 1955), or the end product of an acid-catalysed shift of phosphate from nitrogen to oxygen (Elliott, 1952; Wagner-Jaurreg & Hackley, 1953), is not known.

The experiments described here do not resolve the apparent anomaly, commented upon in the introduction, between the results previously obtained with tissues fixed and degraded under two different conditions, for under both conditions phosphorylserine was present in hydrolysates of the phosphoprotein fraction finally obtained. It would seem that the solution to this and to the other problems mentioned above must await isolation and study of the phosphoprotein itself. In this step, recognition of phosphorylserine as a constituent should prove of some assistance.

SUMMARY

1. Phosphorylserine has been isolated and identified in acid hydrolysates of the phosphoprotein fraction of brain. No other phosphorylated amino acid was detected.

2. The phosphorylserine was found to be radioactive when derived from cerebral slices incubated with glucose in the presence of radioactive inorganic phosphate. The specific radioactivity of the phosphorylserine was increased when derived from slices which had been subjected to electrical pulses for a period of 10 sec.

I am indebted to Professor H. McIlwain for his interest and comments, to Dr J. B. Jepson for a gift of Dowex-50 resin, to Dr G. R. Webster for gifts of phosphorylated amino acids and to Miss Valerie Gooch for competent technical assistance.

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Studies in Detoxication

74. THE METABOLISM OF BENZHYDROL, BENZOPHENONE AND *p*-HYDROXYBENZOPHENONE*

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It is known that many mixed aliphatic aromatic ketones, such as acetophenone, are reduced in the animal body to the corresponding secondary alcohols (Smith, Smithies & Williams, 1954*a*, *b*), but there seems to be no information in the literature concerning the metabolic fate of a purely aromatic ketone, such as benzophenone. A study of the metabolism of this ketone, and its possible metabolites, benzhydrol and *p*-hydroxybenzophenone, was therefore undertaken. *p*-Hydroxybenzophenone is referred to by Schubenko (1893) as being excreted as such by animals, but this appears now to be incorrect for the rabbit. No previous study has been made of benzhydrol, a compound which it has been suggested is a metabolite of the antihistaminic drug, Benadryl (Glazko, McGinty, Dill, Wilson & Ward, 1949; Glazko & Dill, 1949).

Freedlander (1942) has shown that benzophenone and a number of its derivatives have tuberculostatic properties *in vitro*, the highest activity being shown by 2:4′-dichlorobenzophenone. Benzhydrol and *p*-hydroxybenzophenone were less active than benzophenone itself. Benzophenone also has some herbicidal properties (Erickson & Schlesinger, 1954).

* Part 73: El Masri, Smith & Williams (1958).

MATERIALS AND METHODS

Benzhydrol, m.p. 68°, benzophenone, m.p. 49–50°, and *p*-hydroxybenzophenone, m.p. 132°, were commercial samples which were purified. The compounds suspended in water were administered to rabbits by stomach tube. The urine of the animals was analysed for conjugated glucuronic acid and ethereal sulphates by the methods of Paul (1951) and Sperber (1948) respectively.

ISOLATION OF METABOLITES

The glucuronic acid derivatives described below had negative rotations, and by analogy with previously isolated metabolites (Bray, 1953; Teague, 1954) were supposed to be of the β configuration.

From benzhydrol. The urine of rabbits which had received 1 g. of benzhydrol was neutral in reaction and yellow-brown. It did not reduce Fehling's or Benedict's reagent, but when it was heated with dilute acid an oil separated rapidly which quickly turned red. The urine gave an intense naphtharesorcinol reaction for glucuronic acid which developed very rapidly. This latter test indicated the presence of a relatively labile glucuronide. Attempts to isolate this compound showed that it rapidly decomposed if temperatures were not kept low and if prolonged exposures to acid conditions were not avoided during its isolation.

Benzhydrol (1 g.) was fed to each of four rabbits and from the urine, collected for the succeeding 24 hr., the basic lead acetate fraction was prepared in the usual way