Protein-Bound Phosphorylserine in Acid Hydrolysates of Brain Tissue

THE DETERMINATION OF [^{32}P]PHOSPHORYLSERINE BY ION-EXCHANGE CHROMATOGRAPHY AND ELECTROPHORESIS

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1. Partial acid hydrolysates of proteins derived from cortical slices of guinea-pig brain were divided into two parts and fractionated by ion-exchange chromatography and high-voltage electrophoresis. 2. The apparent yield of protein-bound phosphorylserine by the ion-exchange method was about three times that obtained by electrophoresis. 3. The specific radioactivity of phosphorylserine isolated from ^{32}P -labelled slices by electrophoresis was twice that isolated by chromatography. 4. The discrepancies were found to be due to the presence of unlabelled phosphates of unknown composition in the 'phosphorylserine' fraction obtained by the ion-exchange method. 5. Electrical stimulation of slices respiring in the presence of [^{32}P]phosphate increased the specific radioactivity of the total phosphate in the chromatographic 'phosphorylserine' fraction by $53 \pm 11\%$, as compared with only $19 \pm 5\%$ for the phosphorylserine isolated by electrophoresis.

Cation-exchange chromatography on Dowex 50 resin has frequently been used to separate free phosphorylserine from other phosphates in samples of tissue proteins partially hydrolysed by acid (Schaffer, May & Summerson, 1953; Heald, 1958; Ahmed & Judah, 1962; Ahmed, Judah & Wallgren, 1963; Trevor & Rodnight, 1965). During studies of the turnover of protein-bound phosphorylserine in slices of cerebral cortex respiring in the presence of [^{32}P]P_i we had occasion to compare the yield and specific radioactivity of phosphorylserine isolated from partial acid hydrolysates of the slice proteins by ion-exchange chromatography on the one hand and high-voltage electrophoresis on the other hand. A large discrepancy in the results given by the two methods was observed: the yield of phosphorylserine obtained by chromatography was more than twice that obtained by electrophoresis, whereas the specific radioactivity of the chromatographic product was much lower. We have now investigated the reason for this discrepancy and find that it is due to the presence of contaminating phosphates in the 'phosphorylserine' fraction separated by the chromatographic method of Schaffer *et al.* (1953).

EXPERIMENTAL

Preparation and incubation of slices. The conditions used were very similar to those of Trevor & Rodnight (1965). Slices were prepared from guinea-pig cerebral

cortex by a modification of the method of McIlwain & Rodnight (1962) by using a blade and guide, but no incubation fluid to moisten the blade; the slices were picked up directly from either the blade or guide with a flattened rider and weighed. They were then floated in a dish containing 50 ml of oxygenated incubation medium at 37°C and mounted in rapid-transfer holders as described by McIlwain & Rodnight (1962). The incubation medium contained (final concentrations): 128 mM-NaCl; 6.3 mM-KCl; 2.8 mM-CaCl₂; 1.3 mM-MgSO₄; 25 mM-tris-HCl buffer, pH 7.4; 10 mM-glucose. The slices were incubated, labelled with [^{32}P] P_i and electrically stimulated by the procedure used by Trevor & Rodnight (1965) except that at the end of the incubation they were released into 50 ml of ice-cold 10% (w/v) trichloroacetic acid instead of sucrose solution. After being briefly rinsed and drained of excess of fluid they were homogenized in 5 ml of fresh 10% trichloroacetic acid. Control and stimulated slices were carefully paired so that a stimulated slice always had as its control the equivalent cut slice from the opposite hemisphere.

Extraction and hydrolysis of tissue residues. The procedure of Trevor & Rodnight (1965) was used with the addition of a final wash of the protein precipitate with 3 ml of diethyl ether before hydrolysis. Usually homogenates from four slices were pooled before extraction to provide sufficient material for analysis. After hydrolysis with 2 M-HCl for 6 h at 100°C the hydrolysates were freeze-dried over solid KOH. In experiments where a quantitative recovery of phosphates was sought the residues were transferred to silicone-treated conical tubes with about 3 ml of water and the resulting solutions freeze-dried again.

Fig. 1. Fractionation of labelled phosphates on Dowex 50 resin. A protein hydrolysate derived from 350 mg of tissue that had been labelled with ^{32}P as described in the Experimental section was applied to the column and eluted with 0.05 M-HCl at a flow rate of 14 ml/h. The 'phosphorylserine' fraction was eluted between 85 and 100 ml. The first major peak contained P_i and the small peak following the 'phosphorylserine' peak contained phosphorylthreonine.

Chromatography and electrophoresis. Columns (24 cm $\times$ 3 cm 2) of Dowex 50 (X12; H^+ form; 200–400 mesh) were equilibrated with 0.05 M-HCl before use. Samples of protein hydrolysate derived from up to 400 mg of tissue were dissolved in 2 ml of 0.05 M-HCl and applied to the columns with a peristaltic pump. Elution was continued with the same acid at a flow rate of 10–15 ml/h. Fractions were collected every 15 min. In experiments comparing control and stimulated slices the columns were prepared from the same batch of resin and were operated simultaneously from the same pump to ensure uniform conditions. The ^{32}P radioactivity in each fraction was determined by measuring Cerenkov radiation in a liquid-scintillation spectrometer with an efficiency of approx. 40%. The separation of labelled phosphates obtained is shown in Fig. 1. Whatman no. 3MM filter paper was washed chromatographically with 0.05 M-HCl and de-ionized water to decrease the amount of phosphate to about 1 nmol/cm 2 . Samples for electrophoresis were dissolved in approx. 50 μl of 0.05 M-HCl and applied as a band up to 5 cm wide to paper moistened with buffer at pH 1.9 (prepared by adding 50 ml of 98% formic acid to 150 ml of acetic acid and making up to 1 litre with water). Electrophoresis was carried out in a Pherograph Mini 65 apparatus at 2 kV and 100 mA (approx. 50 V/cm) for 90–120 min. The paper was then dried and stained for phosphate by the method of Wade & Morgan (1953). The separation of phosphorylserine from other phosphates is shown in Fig. 2.

Determination of phosphate specific radioactivity. P_i was determined by a micro adaptation of the method of Martin & Doty (1949). The P_i in phosphate-containing areas of paper electrophoresis was determined by first digesting the paper with 12 M- HClO_4 (0.25 ml/5 cm 2) in

Fig. 2. Fractionation of labelled phosphates by electrophoresis. A protein hydrolysate derived from 150 mg of tissue that had been labelled with ^{32}P as described in the Experimental section was applied to Whatman no. 3MM filter paper as a band 3 cm wide. A potential gradient of 50 V/cm for 120 min was used; the pH of the buffer was 1.9. The dried paper was stained for phosphates and scanned for radioactivity. The positions of phosphorylserine and P_i were located by comparison with authentic samples.

the presence of 20 μl of 5% (w/v) ammonium molybdate. The sample was then suitably diluted and P_i determined. Total phosphate in freeze-dried fractions obtained after chromatography was similarly determined.

Radioactivity in the solutions of phosphomolybdic acid in organic solvent used for P_i determination was measured by mixing a portion of the blue solution in a glass vial with an equal volume of ethanol and adding scintillation fluid [0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene]. Counts due to ^{32}P were determined in a liquid-scintillation spectrometer at an efficiency greater than 95%. Radioactive areas of paper after electrophoresis were detected by scanning the paper in an Actigraph III strip scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.).

RESULTS AND DISCUSSION

The time-course of the release of phosphorylserine isolated from the acid hydrolysates by electrophoresis was markedly different from that reported by Trevor & Rodnight (1965), who used ion-exchange chromatography. After hydrolysis for 6 h only about 0.2 μmol of phosphorylserine P/g of tissue was found by electrophoresis as compared with an apparent yield of 0.5–0.7 $\mu\text{mol/g}$ by chromatography (Table 1). Longer hydrolysis times gave very little increase in the yield of phosphorylserine by electrophoresis, whereas the yield found by Trevor & Rodnight (1965) increased to a maximum of 1 $\mu\text{mol/g}$ after 10 h. When the specific radioactivities of phosphorylserine P derived from slices labelled under identical conditions determined by

Table 1. Comparison of phosphorylserine fractions separated by ion-exchange chromatography and electrophoresis

Slices of cerebral cortex (guinea pig) were incubated with ^{32}P , extracted and hydrolysed with 2M-HCl as described in the Experimental section. The acid hydrolysates from 600mg of tissues were combined and divided into two parts. One part was fractionated by chromatography on Dowex 50 and the other by electrophoresis at pH 1.9. The 'phosphorylserine' fraction from the Dowex column was further divided into two parts. On one half total phosphate and radioactivity were determined without further treatment; the other half was concentrated and subjected to electrophoresis at pH 1.9. Total phosphate and radioactivity was determined in the phosphorylserine areas on paper by the procedure given in the text.

	Total phosphate (μmol of P/g of tissue)		Specific radioactivity (c.p.m./ μmol of P)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
'Phosphorylserine' fraction from Dowex column	0.695	0.510	4880	6500
Phosphorylserine isolated by electrophoresis of the hydrolysate	0.195	0.165	11 000	13 000
Phosphorylserine isolated by electrophoresis of the 'phosphorylserine' fraction from the Dowex column	0.164	0.089	10 000	14 500

Fig. 3. Electrophoresis of the 'phosphorylserine' fraction from the Dowex column. Protein hydrolysates derived from 300 mg of ^{32}P -labelled tissue were fractionated on identical Dowex columns in parallel. The 'phosphorylserine' fractions were subjected to electrophoresis at pH 1.9 at 50 V/cm for 90 min and the paper was stained for phosphates and scanned for radioactivity. (a) Phosphates derived from unstimulated tissue; (b) phosphates from stimulated tissue.

using the two methods were compared, that determined by electrophoresis was about twice that determined by the older method (Table 1). This

suggested that the phosphorylserine fraction from the ion-exchange column contained other phosphates of lower specific radioactivity than the phosphorylserine. The fraction containing phosphorylserine from the column was therefore examined by electrophoresis after concentration by freeze-drying. The fraction was found to contain, besides phosphorylserine, two unknown phosphates of higher mobility which were not labelled by ^{32}P (Fig. 3a). The contaminating phosphates did not absorb u.v. light or stain with ninhydrin. They may be sugar phosphates derived from nucleic acids or other cell constituents. Inositol phosphates have a similar mobility, but are considered unlikely candidates since they are labelled by ^{32}P in metabolic experiments *in vitro* (Pumphrey, 1969).

Both Heald (1958) and Trevor & Rodnight (1965) considered the 'phosphorylserine' fraction from the Dowex 50 column to be pure on the basis of finding only a single spot staining with ninhydrin in the position of phosphorylserine on paper chromatograms of concentrates of the fraction. Heald (1958) also eluted the phosphorylserine spot from the paper and found that it had the same specific radioactivity as the phosphate in the 'phosphorylserine' fraction from the column. However, as the paper was not stained for phosphates it is not clear whether the contaminating phosphates were separated from phosphorylserine in the solvent system used.

Specific radioactivity of protein-bound phosphorylserine by the two methods after electrical stimulation. The apparent turnover of protein-bound phosphorylserine in respiring slices is increased by the brief application of electrical pulses to the tissue (Heald, 1957; Trevor & Rodnight, 1965). In the study by

Table 2. *Effect of electrical stimulation on the yield and radioactivity of phosphate in the 'phosphorylserine' fraction from the Dowex column*

Acid hydrolysates of protein from 300–400 mg of tissue were prepared from control and stimulated slices as described in the Experimental section. The hydrolysates from control and stimulated tissue were fractionated on identical columns of Dowex 50 and in each case the 'phosphorylserine' fraction was divided into two parts. The specific radioactivity of the total phosphate was determined in one part and the specific radioactivity of phosphorylserine isolated by electrophoresis on the other part. Values quoted are the means $\pm$ s.e.m. of the numbers of experiments given in parentheses. The mean percentage stimulation was calculated from paired control and stimulated tissues in each experiment and the value for P determined by Student's t test. Total phosphate in the fractions was determined in only three experiments.

	'Phosphorylserine' fraction		Phosphorylserine by electrophoresis	
	Phosphate (μ mol of P/g)	$10^{-2} \times$ Sp. radioactivity (c.p.m./ μ mol of P)	Phosphate (μ mol of P/g)	$10^{-2} \times$ Sp. radioactivity (c.p.m./ μ mol of P)
Control	0.57, 0.56, 0.52	118 ± 5.3 (6)	0.23, 0.21, 0.22	256 ± 20.8 (6)
Stimulated	0.40, 0.47, 0.40	180 ± 13.9 (6)	0.17, 0.22, 0.21	304 ± 24.5 (6)
Stimulation ...	—	$53 \pm 11\%$ (6), $P < 0.01$	—	$19 \pm 5\%$ (6), $P < 0.01$

Trevor & Rodnight (1965), in which the ion-exchange method was used to isolate phosphorylserine, the increase on stimulation of phosphorylserine specific radioactivity was about 50%. A similar value of $53 \pm 11\%$ was found in the present work with the chromatographic method; however, in the same series of experiments the increase on stimulation of the radioactivity of phosphorylserine isolated by electrophoresis from the column 'phosphorylserine' fraction was only $19 \pm 5\%$ (Table 2). The greater apparent increase in the specific radioactivity of the 'phosphorylserine' in the original experiments was not due to increased labelling of the unknown phosphates because they remained unlabelled in the stimulated samples as well as in the control samples (Fig. 3); also, the radioactivity present in the small amount of material remaining on the origin was too small to affect the final result. Since we have shown in other experiments (and illustrated in Table 2) that the yield of electrophoretically pure phosphorylserine does not change significantly on stimulation, there would appear to be only one explanation for the discrepancy in the two methods: a lower yield of one or both of the unknown phosphates in the stimulated tissues. A direct determination of the unknown phosphates was not attempted, but in the three experiments of Table 2 where phosphates were determined quantitatively the total phosphate recovered in the 'phosphorylserine' fraction from the column was less in the stimulated samples by an amount more than adequate to account for the

greater proportional increase in specific radioactivity in the samples analysed by chromatography.

The finding that the 'phosphorylserine' fraction used by Trevor & Rodnight (1965) contains a mixture of phosphates throws some doubt on the interpretation of their quantitative data. In particular their conclusion that the increase in phosphorylserine radioactivity induced by stimulation is confined to phosphoproteins occurring in the membrane fraction needs reinvestigation, since it is not known whether the yield of unknown phosphates in the different fractions is the same.

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REFERENCES

- Ahmed, K. & Judah, J. D. (1962). *Biochim. biophys. Acta*, **57**, 245.
- Ahmed, K., Judah, J. D. & Wallgren, H. (1963). *Biochim. biophys. Acta*, **69**, 428.
- Heald, P. J. (1957). *Biochem. J.* **66**, 659.
- Heald, P. J. (1958). *Biochem. J.* **68**, 580.
- McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry*, p. 145. London: J. and A. Churchill Ltd.
- Martin J. B. & Doty, D. M. (1949). *Analyt. Chem.* **29**, 965.
- Pumphrey, A. M. (1969). *Biochem. J.* **112**, 61.
- Schaffer, N. K., May, S. C. & Summerson, W. H. (1953). *J. biol. Chem.* **202**, 67.
- Trevor, A. J. & Rodnight, R. (1965). *Biochem. J.* **95**, 889.
- Wade, H. E. & Morgan, D. M. (1953). *Nature, Lond.*, **171**, 529.