The Separation of Nitrogen-Containing Phosphate Esters from Brain and Spinal Cord by Ion-Exchange Chromatography

By R. W. R. BAKER AND G. PORCELLATI*

Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

(Received 21 April 1959)

The quantitative behaviour of ion-exchange resins in the analysis by column chromatography of mixtures of amino acids (Moore & Stein, 1951) is already well established. Microbead sulphonated polystyrene resins have been used for analysing protein hydrolysates (Askonas, Campbell, Godin & Work, 1955; Van der Schaaf & Huisman, 1955; Jacobs, 1956*a*). Similar methods were employed in the study of phosphorus-containing peptides present in hydrolysed protein by Flavin (1954) and by Schaffer, Harshman & Engle (1955); Tallan, Moore & Stein (1954) used them for separating phosphorylethanolamine and glycerylphosphorylethanolamine from tissue extracts. Apart from the last-mentioned work the technique has, however, been applied but little in the study of nitrogen-containing phosphate esters, and in particular no report on the analysis by this means of phosphorylcholine and its derivatives has yet appeared.

In a study of protein-free aqueous extracts of hen brain and spinal cord we have characterized some of the peaks obtained by chromatography and have compared the two tissues. This paper is concerned primarily with peaks containing both nitrogen and phosphorus. In addition to analysis of fractions of eluate by the ninhydrin reaction, they were analysed for phosphorus and choline. Glycerylphosphorylcholine and phosphorylcholine were distinguished by the fact that the former is rapidly hydrolysed by acid, whereas the latter requires more vigorous conditions.

The presence of phosphorylserine, glycerylphosphorylethanolamine and phosphorylethanolamine in brain and spinal cord, and of phosphorylcholine, glycerylphosphorylcholine and of other phosphorus compounds in brain, has been confirmed; amounts of some of these have been measured. The chromatographic behaviour of some free phosphate esters has been studied quantitatively.

EXPERIMENTAL

Materials

Ion-exchange resin. Zeo-Karb 225, 4.5% cross-linked (water-regain 1.8 g./g.), 200–400 mesh, (The Permutit Co. Ltd.) was washed with 10 vol. of water and graded by

stirring with water (5 vol.) and allowing about 5% (v/v) of the resin to settle. The remaining suspension was decanted and 95% of the solid allowed to settle out in about 15 hr.; the cloudy supernatant was discarded. The portion of the resin selected corresponded to 85–90% (v/v) of the original material. Dr S. Jacobs kindly examined the resulting resin and reported a range in bead diameter of 10–50 μ . It was converted into the sodium form by treatment with 2*N*-NaOH (2 vol.), separated by filtration under reduced pressure and washed free of excess of alkali with five changes of water.

For use, the resin was washed with 0.5 *N*-NaOH and then equilibrated with 0.2 *M*-sodium citrate buffer, pH 5.1 (see below), by suspending twice in 3 vol. followed by sedimentation and rejection of the supernatant liquid.

Detergent. The polyoxyethylene lauryl ether BRIJ 35 (L. Light and Co. Ltd.) was used in some experiments.

Thiodiglycol. This compound (British Drug Houses Ltd.) was redistilled under reduced pressure, the middle third of the distillate (b.p. 168°/14 mm.) being collected.

Buffer solutions. (1) Sodium citrate, 0.2 *M*, pH 2.2: citric acid 42 g.; NaOH 16.8 g.; conc. HCl 32 ml.; to 2 l. with water. (2) Sodium citrate 0.2 *M*, pH 3.1: citric acid 71.4 g.; NaOH 28.2 g.; conc. HCl 39.3 ml.; to 3.4 l. with water. (3) Sodium citrate 0.2 *M*, pH 5.1: citric acid, 4.2 g., *N*-NaOH 40 ml.; to 200 ml. with water.

pH values were measured with a glass electrode. Each buffer contained 0.5% of thiodiglycol and 0.01% of detergent, and was stored at 3°, but in experiments in which fractions were to be hydrolysed with HCl the thiodiglycol was omitted (Hirs, Moore & Stein, 1956). No detergent was used in experiments in which choline was estimated, since the former interfered in the iodine method.

Methods

Chromatographic methods. Buffered resin, suspended in its own volume of buffer solution (3), was poured in four portions into a vertical glass tube (12.5 mm. bore) fitted with a sintered-glass diaphragm at its lower end to give a column 166 cm. long. The column settled under gravity to a volume of 210 ml., as solution ran off through the sintered glass until the liquid was level with the top of the resin. Before applying the sample material, 0.2 *N*-NaOH (100 ml.), followed by at least 250 ml. of buffer (1), was passed through under a pressure (20 cm. Hg) of nitrogen. Both solutions generally contained detergent and thiodiglycol as above. When the liquid was down to bed level, the sample (2–3 ml. adjusted to pH 2.0–2.5) was poured on and drained to bed level. Fractionation of the mixture was then carried out at 22° by passing citrate buffer (2) under a pressure (20–25 cm. Hg) of nitrogen, the arrangement being shown in Fig. 1. Effluent (about 500 ml.) was

* British Council Scholar.

collected in 2 ml. fractions, the flow rate being 5 ml./hr. The column was then regenerated *in situ* with NaOH and buffer (1) as described above.

For examination of peaks detected as described below, the component fractions were pooled and two-dimensional chromatography on paper was applied as reported earlier (Porcellati, 1958). Where necessary, removal of cations from solutions (Jacobs, 1956*b*) was carried out by passing them under pressure (10 cm. Hg.) of nitrogen through columns (5 cm. long, 1 cm. diam.) of Zeo-Karb 225 microbead resin in the free-acid form, washed *in situ* with water. After adsorption, the column was washed free of acids with water and then eluted with aq. 0.2*N*-NH₃ soln. (45 ml.). Eluates were distilled under reduced pressure to 2–3 ml. and evaporated to dryness on a water bath. Residues were taken up in 0.1*N*-HCl for application to paper. Washing of the resin before and after adsorption was controlled by measuring the conductivity of effluent with an alternating-current bridge and platinized electrodes; generally 50 ml. of water was required for each wash.

Analysis of effluent. (1) *Ninhydrin reaction.* The method of Moore & Stein (1954), modified as described below, was used with all or part of each fraction tested, water being used to bring the sample to 2 ml. As standard, leucine (0.2 μ mole/2 ml. in citrate buffer, pH 5.1) was used. The original preparation (Moore & Stein, 1954) of the ninhydrin-hydrindantin reagent was modified (Jacobs, 1956*b*) in that the solution of ninhydrin (AnalaR) and hydrindantin in methyl Cellosolve (2-methoxyethanol) was treated with its own volume of Zeo-Karb 225 (H) to remove ammonia and trace metals and stored over the resin in an atmosphere of nitrogen before mixing with acetate buffer. Blank values were reduced to a uniform low value as a result.

(2) *Choline.* After hydrolysis of 1 ml. from the fraction in 2*N*-HCl for 1 hr. under reflux, or for 48 hr. in a sealed tube at 123°, the solution was evaporated to dryness on a water bath, with further evaporation, after addition of 3 ml. of water, to remove HCl. The residue was dissolved in 5%

(w/v) trichloroacetic acid and choline was determined by the periodate method (Webster, 1956).

(3) *Phosphate.* The method of Ernster, Zetterström & Lindberg (1950) as modified by Strickland, Thompson & Webster (1956) was used.

Tissue extracts. Hens of mixed breed were decapitated and the brains and spinal cords were removed quickly and cooled on ice. Weighed amounts of tissue, finely minced and homogenized in 2–3 vol. of ice-cold water, were deproteinized by bringing to pH 4.8–5.0 with 0.5*N*-HCl and pouring into 20 vol. of boiling water. The precipitate, filtered off after 2–3 min., was washed twice with hot water. The combined filtrate and washings were concentrated under reduced pressure to about 10 ml. and dialysed at 4° for two 12 hr. periods against 100 ml. of water with continuous agitation. The combined dialysates were concentrated to 2–3 ml. under reduced pressure, adjusted to pH 2.0–2.5 with 0.1*N*-HCl and applied to the ion-exchange column. The extract applied corresponded to 2.0–2.5 g. of fresh tissue.

RESULTS

The results of chromatography of extracts of brain and cord are shown in Figs. 2 and 3, where the extinctions after reaction with ninhydrin, calculated as μ moles of leucine/2 ml. of fraction, are plotted against the serial numbers of the fractions taken. After identification of some of the peaks, the colour yields with ninhydrin of the substances involved were determined and approximate closely to published data (Moore & Stein, 1954). This enabled the absolute amounts of the substances to be calculated (Table 1). Collection of the eluate was continued to a total volume of about 500 ml., since only the relatively acidic phosphate esters were being studied. Figs. 2 and 3 give a direct comparison of brain with spinal cord. The latter gave, in fractions 108–111, 113–118 and 146–148, minor constituents which were not found in the brain, whereas a small fraction (33–36) in brain was not found in the cord; none of these fractions was examined since insufficient material was obtained. The other differences observed are in the quantities present, and in particular the fraction 138–141, abundant in cord, is a relatively small component of brain (135–139).

Preliminary chromatography on a shorter column (140 cm.) of a mixture of three amino acids had shown, as discussed below, that our fraction numbers corresponded closely with the eluate volumes reported by Tallan *et al.* (1954), and that recoveries were quantitative. Provisional identification of our peaks was therefore made by reference to the results of these authors, and suitable fractions were chosen for further study. They were desalted with ion-exchange resin and examined by paper chromatography by the methods described above.

Analysis for phosphorus and examination of the materials by paper chromatography, both as obtained and after hydrolysis, showed that peak

Fig. 1. Apparatus for ion-exchange chromatography (schematic). A and A' are diaphragms of sintered glass.

Table 1. Colour yields with ninhydrin and amounts of certain compounds in avian nervous tissue

Substance	Colour-yield/ μ mole (relative to leucine = 100)	Amounts in protein-free aqueous extracts of avian nervous tissue (mg./100 g. of fresh tissue)							
		Brain				Spinal cord			
		No. of determinations	Range	Mean	s.d.	No. of determinations	Range	Mean	s.d.
Phosphorylserine	74	9	0.6-2.3	1.36	0.56	4	0.6-1.2	0.95	0.26
Phosphorylethanolamine	44	10	7.5-31.8	19.6	0.60	4	0.6-7.8	6.9	0.79
Glycerolphosphorylethanolamine	52	9	11.6-26.0	17.1	5.46	4	12.3-14.1	13.1	0.83
Taurine	90	9	14.3-30.7	18.5	5.18	4	4.4-7.1	6.1	1.23
Aspartic acid	95	9	27.1-53.8	38.6	9.50	2	24.1-29.6	26.9	—
Threonine	94	8	6.1-11.2	7.8	1.62	2	5.8-6.2	6.0	—
Glycerolphosphorylcholine	0	10	1.9-7.9	5.2	2.08	3	1.9-4.8	3.2	1.47
Phosphorylcholine	0	9	12.0-17.2	14.1	1.83	3	7.0-8.1	7.5	0.56

Fig. 2. Chromatogram of extract from 2.5 g. of hen brain. —, Leucine equivalent; - - -, choline in hydrolysed fractions. 1, Phosphorylserine; 2, glycerylphosphorylethanolamine; 3, glycerylphosphorylcholine; 4, phosphorylethanolamine; 5, phosphorylcholine; 6, taurine; 7, urea; 8, unknown; 9, peptide; 10, aspartic acid; 11, threonine; 12, serine.

Fig. 3. Chromatogram of extract from 2.1 g. of hen spinal cord. —, Leucine equivalent; - - -, choline in hydrolysed fractions. For significance of numbered peaks see caption to Fig. 2.

45-49 (44-46 in cord) was phosphorylserine. The molar ratio amino nitrogen:P was 1.10 (calc. 1.00). Peak 61-71 (61-69 in cord) was identified as glycerylphosphorylethanolamine by chromatography of the substance alone and after admixture with the authentic material. Chromatograms of the hydrolysed materials (2N-HCl; 2 hr.; 100°) revealed the presence of glycerophosphate, ethanolamine and a small amount of inorganic phosphate. The molar ratio amino nitrogen:P was 0.89, a low value which may be interpreted as due to contamination of the substance with glycerylphosphorylcholine.

The major component 82-87 (82-88 in cord) was found to be phosphorylethanolamine. After hydrolysis (6N-HCl; 48 hr.; 120°; sealed tube), evaporation and desalting, analysis on paper chromatograms showed the presence of ethanolamine, inorganic phosphate and a small amount of unchanged phosphorylethanolamine. The molar ratio amino nitrogen:P found was 0.98 for the peak material. The unidentified minor peak (33-36) was of high phosphorus content, amino nitrogen and P being present in approximately equimolar amounts.

Brain fractions (37-41) gave after hydrolysis a deeper colour with ninhydrin, and on paper chromatography six ninhydrin-reacting spots were revealed, and this peak is therefore considered to be of peptide-like composition; no phosphorus was detected. Another component (fractions 135-139 in brain and 138-141 in cord) showed similar behaviour but contained phosphorus. The identity of these peaks was confirmed by paper chromatography with and without hydrolysis, by analysis for phosphorus and by the pentose reaction with orcinol. Although in brain the quantity of this fraction is such that only 44 µg. of P is present in 100 g. of wet tissue, the amount in spinal cord is very much larger (more than 0.7 mg. of P/100 g. of tissue). The substance was hydrolysed only slowly in 6N-HCl (sealed tube; 120°) and was still detectable after 48 hr. The absorption found in the ninhydrin reaction with hydrolysate was about 6.4 times that obtained with the parent peptide. Among the five or six major products of hydrolysis resolved on paper chromatograms, provisional identifications of phosphorylserine, phosphorylethanolamine, glutamic acid and valine have been made. The original substance gave a green in the orcin reaction for pentose.

In addition, some of the more prominent ninhydrin-reacting fractions from brain not containing P were identified as taurine (91-100), aspartic acid (206-222), threonine (223-233) and serine (241 *et seq.*). The prominent phosphorus-free component (fractions 131-133) and a lesser one (178-182) remain unidentified.

The presence of phosphate esters of choline in

the brain extract was confirmed in further work with similar columns. Fractions of eluate were analysed by hydrolysing and then determining the choline liberated. This was necessary since choline, in the quantities treated, does not give any colour with ninhydrin, a fact that confers some specificity. Two fractions containing choline were found (Fig. 2). The peak (65-71) liberated all its choline after hydrolysis (6N-HCl; 100°) for 1 hr., as is found with glycerylphosphorylcholine, in contrast with phosphorylcholine, which is hydrolysed only under the more vigorous conditions described below (Dawson, 1955). The position of the peak corresponded with that found when authentic glycerylphosphorylcholine was run chromatographically in the same way. The pooled fractions of the peak from brain extract, after desalting, were examined by paper chromatography in two directions, with spraying to reveal esterified phosphate (Bandurski & Axelrod, 1951); the result confirmed the identity of the glycerylphosphorylcholine and revealed the presence in the same fraction of glycerylphosphorylethanolamine, as had been found earlier. Control experiments showed that the latter, in a quantity such as that present, did not influence the determination of choline by the method used, although excess of glycerylphosphorylethanolamine (2 mg.), when hydrolysed as described above, gave a faint final magenta pink in CHCl_3 , clearly different from the red-orange given by choline. Since the two substances are resolved poorly if at all by the column, analysis for P is equivocal.

The second peak in which choline was detected (85-91, Fig. 2) was identified as phosphorylcholine, which yields the base quantitatively only on hydrolysis for 48 hr. (6N-HCl; 120°; sealed tube). Two-dimensional chromatograms of the unhydrolysed pooled desalted material, sprayed to reveal esterified phosphorus, showed a spot corresponding to that for authentic phosphorylcholine. The choline:P molar ratio was 1.04:1. Chromatography on the column of pure phosphorylcholine gave a peak in the same position, revealed by analysis both for P and for choline. Our result indicates 12.0-17.2, mean 14.1 mg./100 g., as the phosphorylcholine content of brain. Taurine, urea, HCl and citrate buffer were shown in control experiments not to interfere with the estimation of esterified choline by the hydrolysis and colorimetric methods employed. Recoveries of 98 and 99% for phosphorylcholine and glycerylphosphorylcholine respectively were recorded when a mixture of 2 µmoles of each of the pure compounds was run on the column, followed by estimation of both P and choline in the effluent. No free choline was detected in the eluates examined, as would be expected since such a basic substance would appear only at much greater eluate volumes.

Hen-brain extract from one bird only was analysed directly for choline compounds, without acid treatment and after hydrolysis for 1 hr. or 48 hr. as described above (Dawson, 1955). This differential hydrolysis indicated that 100 g. of the tissue contained 2.4 mg. of 'free choline', 2.1 mg. of glycerylphosphorylcholine and 9.8 mg. of phosphorylcholine. The value for free choline obtained by the iodine method with an extract containing so many substances should be taken only as provisional.

DISCUSSION

The chromatographic method described appears to be well suited, in conjunction with specific colorimetric methods, for the determination not only of amino acids, but of phosphate esters of choline, ethanolamine and serine in extracts of brain or spinal cord. Sharp peaks were obtained with each of the various classes of substance, ranging from those of relatively small molecular weight to others of grosser structure resembling peptides. Some overlapping did occur, as, for example, with the glycerylphosphoryl derivatives of ethanolamine and choline, but here confusion was avoided by the differential use of specific methods of analysis.

The value obtained by Tallan *et al.* (1954) by column chromatography for the amounts of phosphorylethanolamine (41.9 mg./100 g.) in cat-brain extracts is greater than the quantities found in rat brain by other authors (12 mg./100 g., Awapara, Landua & Fuerst, 1950; 9 mg./100 mg., Ansell & Dawson, 1952; 22 mg./100 mg., Ansell & Richter, 1954) who relied on paper chromatograms. With hen brain, there was reasonable agreement found in this Laboratory between the values from the column and results with paper chromatography. Thus for phosphorylethanolamine a quantity of 19.6 mg./100 g. of brain was found with the column, and 13.8 mg./100 g. by the paper method (Porcellati, 1958). For phosphorylcholine also the results with the column (12.0–17.2 mg./100 g.) are fairly close to those obtained by paper chromatography (9.2 mg./100 g., Porcellati, 1958). These values quoted from analysis on papers have not been corrected for the loss that is known to occur. Whereas recoveries in two-dimensional chromatography are known to be between 75 and 85 %, the column analysis is much more quantitative, as shown, for example, by recoveries of 17 amino acids (Moore & Stein, 1954), and as found in the present work with phosphate esters of choline. Lower quantities of phosphorylserine were, however, found in brain by the column method (1.36 mg./100 g.) than by paper chromatography (4.3 mg./100 g.) (Porcellati, 1958). This discrepancy can probably be attributed to the superior resolving power of the column, for on the paper the

phosphorylserine may be contaminated with peptides.

Since this paper was written, there has appeared a report (Okumura, Otsuki & Aoyama, 1959) of analysis of hen-brain extract on Dowex resin. Values found for glycerylphosphorylethanolamine (24.9 mg./100 g. of tissue) and aspartic acid (33.4 mg./100 g.) fall within our range; values for phosphorylethanolamine (45.8 mg./100 g.) and taurine (4.4 mg./100 g.) are higher whereas the amount of threonine (4.2 mg./100 g.) is lower than that found in the present investigation.

SUMMARY

1. Deproteinized aqueous extracts of avian brain and spinal cord have been resolved by chromatography on a column of a finely divided sulphonated polystyrene resin and the more mobile components examined.

2. The method is shown to be suitable for the separation and quantitative estimation of phosphorylcholine and glycerylphosphorylcholine in brain and spinal cord.

3. Phosphorylserine was well resolved. Phosphorylethanolamine and glycerylphosphorylethanolamine, although overlapping to some extent their choline counterparts, could be determined by specific reaction with ninhydrin and the quantities present in brain were ascertained.

The authors wish to thank: Professor R. H. S. Thompson for his interest and provision of facilities; Dr G. R. Webster for gifts of phosphorylserine, phosphorylethanolamine, phosphorylcholine and glycerylphosphorylcholine; Dr R. M. C. Dawson for a gift of glycerylphosphorylethanolamine and Dr S. Jacobs for helpful discussion on the methods and on the properties of the resin used. They are also grateful to the British Council for a grant to one of us (G.P.).

REFERENCES

- Ansell, G. B. & Dawson, R. M. C. (1952). *Biochem. J.* **50**, 241.
- Ansell, G. B. & Richter, D. (1954). *Biochem. J.* **57**, 70.
- Askonas, B. A., Campbell, P. N., Godin, C. & Work, T. S. (1955). *Biochem. J.* **61**, 105.
- Awapara, J., Landua, A. J. & Fuerst, R. (1950). *J. biol. Chem.* **183**, 545.
- Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
- Dawson, R. M. C. (1955). *Biochem. J.* **60**, 325.
- Ernster, L., Zetterström, R. & Lindberg, O. (1950). *Acta chem. scand.* **4**, 942.
- Flavin, M. (1954). *J. biol. Chem.* **210**, 771.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1956). *J. biol. Chem.* **219**, 623.
- Jacobs, S. (1956a). *Rés. Comun. 11^e Congr. Quim. anal., Lisbon*, p. 212.

- Jacobs, S. (1956b). *Analyst*, **81**, 502.
 Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
 Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
 Okumura, N., Otsuki, S. & Aoyama, T. (1959). *J. Biochem., Tokyo*, **46**, 207.
 Porcellati, G. (1958). *J. Neurochem.* **2**, 128.
 Schaffer, N. K., Harshman, S. & Engle, R. R. (1955). *J. biol. Chem.* **214**, 799.
 Strickland, K. P., Thompson, R. H. S. & Webster, G. R. (1956). *J. Neurol. Psychiat.* **19**, 12.
 Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
 Van der Schaaf, P. C. & Huisman, T. H. J. (1955). *Biochim. biophys. Acta*, **17**, 81.
 Webster, G. R. (1956). *Biochim. biophys. Acta*, **20**, 432.

The Phosphate Esters of Mammalian Skin Maintained on Glucose and Various Deoxyglucoses

By SHEILA A. BROOKS, J. C. LAWRENCE AND C. R. RICKETTS

*Medical Research Council Industrial Injuries and Burns Research Unit,
 Birmingham Accident Hospital, Birmingham, 15*

(Received 23 April 1959)

In most living cells the first step in glycolysis is phosphorylation of glucose at C-6, catalysed by the enzyme hexokinase. Studies with hexokinase preparations from yeast and from brain show that compounds closely related to glucose may also be phosphorylated, e.g. 1:5-anhydro-D-glucitol, 2-deoxy-D-glucose, D-glucosone, D-glucosamine and D-mannose (Harpur & Quastel, 1949; Brown, 1951; Johnstone & Mitchell, 1953; Sols & Crane, 1954; Woodward & Hudson, 1955), but 3-deoxy-D-glucose, N-acetyl-D-glucosamine and D-galactose are not phosphorylated (Grant & Long, 1952; Sols & Crane, 1954). Of these compounds 2-deoxyglucose, glucosone and glucosamine are known to interfere with tumour-cell glycolysis (Woodward & Hudson, 1954; Yushok & Batt, 1957). 2-Deoxyglucose is also reported to inhibit the synthesis of influenza virus in chick embryos (Kilbourne, 1959), presumably by functioning as an antimetabolite of glucose. Wick, Drury, Nakada & Wolfe (1957) concluded that the inhibition of glycolysis which occurred when 2-deoxyglucose was given to rabbits was at the phosphoglucisomerase stage of glycolysis. Other than this there seems to be very little published information about the effects of deoxy-sugars on normal mammalian cells and further experiments seemed desirable. Tissue culture affords a convenient approach to this problem and in the experiments to be described slices of mammalian skin were maintained on media containing 1:5-anhydro-D-glucitol (which may be regarded as 1-deoxy-D-glucose), 2-deoxy-D-glucose and 3-deoxy-D-glucose (Fig. 1). D-Glucosone, D-mannose and D-glucosamine were also included since these hexoses differ from glucose at C-2.

The metabolism of the skin cells has been studied by micro-respirometry and by chromato-

graphy and radioautography of the phosphate esters formed during maintenance on radioactive inorganic phosphate. It was first necessary to identify the phosphate esters of skin maintained on glucose and then to examine skin maintained on deoxy-sugars. For this purpose certain phosphate esters

Fig. 1. Structural formulae of deoxyglucoses.