ANION-EXCHANGE CHROMATOGRAPHY OF SUGAR PHOSPHATES WITH TRIETHYLAMMONIUM BORATE

MARIA J. LEFEBVRE*, NELIDA S. GONZALEZ** AND HORACIO G. PONTIS***

Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales§, Buenos Aires (Argentina)

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Several methods of separation of sugar phosphates and other phosphoric esters by ion exchange chromatography have been described[1–6]. The recovery of the sugar phosphates after most of these separations is troublesome and time consuming, which diminishes the effectiveness of the technique and its application to microscale use.

This paper describes a method which allows separation of most of the sugar phosphates on an anion-exchange resin column and permits their almost quantitative recovery. It is based on the use of linear gradient elution with ammonium or triethylammonium borate and on the removal of these salts after freeze-drying by distillation with methanol.

EXPERIMENTAL

Materials

Fructose-1-P was synthesized according to RAYMOND AND LEVENE[7]. Fructofuranose-2-P and fructopyranose-2-P were prepared according to PONTIS AND FISCHER[8]. Mannose-1-P was kindly given by Dr. A. MORENO and galactose-1-P, N-acetylglucosamine-1-P and N-acetylgalactosamine-1-P were generously provided by Dr. C. E. CARDINI. All the other sugar phosphates were commercial samples.

Analytical procedures

The following analytical methods were used: BARTLETT's[9] method for phosphate, the anthrone method[10] for reducing power, ROE's[11] method for fructose and REISSIG *et al.*'s[12] method for acetylhexosamines.

Column chromatography

Dowex-1 X4 resin (200–400 mesh) was used for the chromatography of the hexose phosphates. Dowex-1 (Cl^-) resin was converted into the borate form by passing 0.8 *M* potassium tetraborate until all the chloride had been displaced. It was then washed with water until the effluent gave no precipitate on addition of silver nitrate.

The columns used were 60 cm long, 0.5 cm in diameter and were prepared by allowing a suspension of resin to settle until a bed 45 cm high was obtained. Consider-

* On leave of absence from Tucuman University, Tucumán, Argentina.
** Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.
*** Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.
§ Obligado 2490, Buenos Aires (28).

able shrinkage occurred during the chromatography. Therefore, the columns were disassembled after each run, and the resin washed again with 0.8 *M* potassium tetraborate and water as above. The resin could be reused many times, except for a layer at the top which became dark during the run and which was replaced every time with fresh resin.

The ammonium[8] or triethylammonium tetraborate used for elutions was prepared by mixing a freshly made boric acid solution with ammonia or triethylamine. Thus, a 0.4 *M* triethylammonium tetraborate was prepared by dissolving 99.2 g of boric acid and 112 ml of triethylamine in water and making the volume up to one liter.

The mixture of sugar phosphates to be separated was adjusted to pH 8 with ammonium hydroxide and applied to the column. After washing the column with water the sugar phosphates were eluted with a linear gradient of ammonium or triethylammonium borate. Fractions of 1.4 ml were collected at a flow rate of 1.0–1.5 ml/min. Aliquots of every second fraction were taken for analysis.

Columns of the size indicated above could be used for separation of 2 to 100 μmoles of a complex mixture of sugar phosphates.

Recovery

The fractions under each peak were pooled and freeze dried. When ammonium borate was used as eluant, a very light powder remained which was easily removed by two or three evaporations to dryness with methanol. With triethylammonium borate a residue that stuck to the wall of the flask was left and made removal with methanol slower. Freeze drying can be replaced by evaporation to dryness in a rotary evaporator when ammonium borate has been used as eluant.

The sugar phosphates thus freed from salts were dissolved in water and adjusted to pH 7.

Radioactivity measurements

When radioactive samples were under investigation, suitable aliquots from the column fractions were plated on aluminum disks and evaporated to dryness on a water bath. Methanol was then carefully added taking care not to overflow the planchet. Depending on the aliquot, two or three methanol additions were needed to eliminate all the salt. With aliquots of 0.5–1.0 ml it was found convenient to treat the planchet perimeter with silicon grease, in order to keep the liquid centered.

Counting was carried out with a gas flow counter (Tracerlab, Inc.).

RESULTS AND DISCUSSION

The commonly occurring monophosphorylated sugars have nearly identical dissociation constants[13]. This makes their complete separation by simple ion-exchange unlikely.

The use of borate, introduced by KHYM AND COHN[1] permitted the separation of glucose-1-P, glucose-6-P, fructose-6-P and ribose-5-P from each other and from fructose-1,6-P_2. KHYM AND ZILL[14] had used relatively concentrated solutions of borate for the separation of neutral sugars, but for phosphoric esters, the use of dilute borate on the basis of the following reasoning[1] was decided upon. "Since borate ion exists only in alkaline solutions where phosphate esters are doubly ionized and

hence more strongly bound to anion exchangers, the use of borate as the replacing ion as well as the complex-forming ion involves higher concentrations than are compatible with easy recovery of the separated esters". Hence, KHYM *et al.*[2] developed methods by which borate complexes of phosphate esters could be separated in chloride or sulfate systems containing only sufficient borate to allow complexing. However, this did not simplify the recovery procedure, as the removal of the eluting salts required the use of cation and anion exchangers[2] or solvent extraction[6].

The problem of recovery can be overcome by replacing potassium or sodium borate as eluants with ammonium or triethylammonium borate. These salts are easily removed by freeze-drying and distillation with methanol. In this way sugar phosphates can be freed from borate in concentrations up to 0.5 *M*. Ammonium borate has already been used as an eluant by PONTIS AND FISCHER[8] for the separation of fructose 2-phosphates from fructose-1-P. It has now been found that the use of triethylammonium borate as eluant gives more reproducible results for complex separations. Hence, this salt is generally used, particularly for analytical runs. Ammonium borate is employed when substances are easily separated, especially for preparative runs on account of its easier removal (see Experimental).

The application of this technique permits almost quantitative recovery of the sugar phosphates (Table I). Moreover, if BARTLETT's[9] procedure for phosphate de-

TABLE I

AMOUNT OF SUGAR PHOSPHATES RECOVERED

Sugar phosphate	*μmoles added to column*	*Percent recovery*	*μmoles before freeze-drying**	*Percent recovery*
N-Acetylglucosamine-1-P	2.3	93	1.65	86
N-Acetylgalactosamine-1-P	7.0	95	5.7	93
Glucose-1-P	2.7	97	2.2	95
Galactose-1-P	12.0	105	6.0	92
Mannose-1-P	9.0	98	7.2	94
Fructose-6-P	7.3	98	6.7	91
Mannose-6-P	15.0	94	3.0	93
Fructose-1-P	8.0	94	7.2	84
Glucose-6-P	9.6	91	5.0	100
Galactose-6-P	9.0	97	8.0	96
Fructose-1,6-P_2	9.0	94	6.4	81

* Only part of the ester recovered after the column was submitted to freeze-drying.

termination is employed, the method can be used with as little as 0.2 μmole of aldose-1-phosphates and fructose-2-phosphates. A further advantage is that these acid labile esters are not exposed to strong acid conditions during their isolation.

The results obtained with the different sugar phosphates are shown in Figs. 1 and 2. Similar elution patterns have been obtained by GOODMAN *et al.*[3] and DIEDRICH AND ANDERSON[6]. However, the triethylammonium borate system offers the advantage that the procedure is simpler and that the substances do not become so dilute.

The separation between fructose-6-P and glucose-6-P is complete (Fig. 1A). A simple procedure is thus available for the purification of fructose-6-P. The triethylammonium borate gradient used for the more common sugar phosphates also resolves mixtures of fructose-2-phosphates and fructose-1-P (Fig. 1C). On the other hand, the

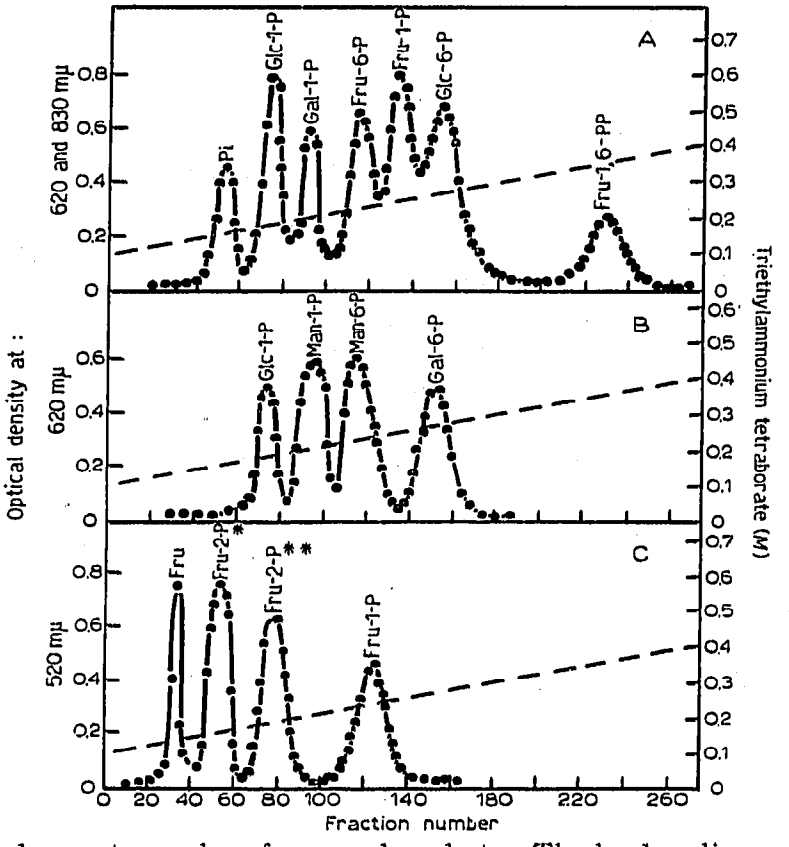

Fig. 1. Ion-exchange chromatography of sugar phosphates. The broken line represents the gradient from 0.1 *M* to 0.4 *M* triethylammonium tetraborate (360 ml total volume). The mixtures applied to the column were as follows, in *μ*moles: [A] P_i (2); glucose-1-P (9); galactose-1-P (7); fructose-1-P (10); fructose-6-P (10); glucose-6-P (17); fructose-1,6-P_2 (9). [B] Glucose-1-P (5); mannose-1-P (18); mannose-6-P (17); galactose-6-P (20). [C] Fructose (1); fructofuranose-2-P (Fru-2-P*, 3); fructopyranose-2-P (Fru-2-P**, 4); fructose-1-P (6). Assays were as follows: anthrone for sugar phosphates in A and B, ROE's[11] method for fructose compounds in C, and BARTLETT's[9] method for inorganic phosphate.

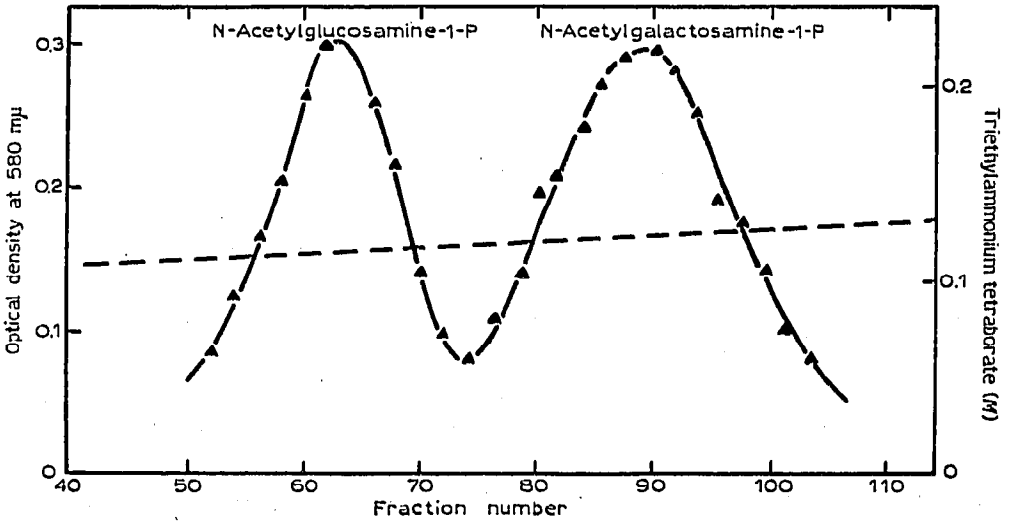

Fig. 2. Ion-exchange chromatography of N-acetylhexosamine 1-phosphates. The broken line represents the gradient from 0.1 *M* to 0.14 *M* triethylammonium tetraborate (200 ml total volume). The mixture applied to the column contained N-acetylglucosamine-1-P (2 *μ*moles) and N-acetylgalactosamine-1-P (5 *μ*moles). Assay according to REISSIG *et al.*[12] after acid hydrolysis.

separation of N-acetylglucosamine-1-P and N-acetylgalactosamine-1-P can be achieved as shown in Fig. 2, by employing a less steep gradient than that used for the other esters.

The use of ammonium or triethylammonium borate as eluant is also advantageous when dealing with radioactive samples. In this case, suitable aliquots from the column fraction can be plated directly on aluminum disks, evaporated to dryness on a water bath, and treated with methanol until no salt residue is left. Self-absorption owing to the presence of salts is completely eliminated. In the chromatogram shown in Fig. 3, fructose-1-P-^{14}C, 36,000 counts/min, was applied together with the esters indicated. After plating and counting, 37,000 counts/min were recovered.

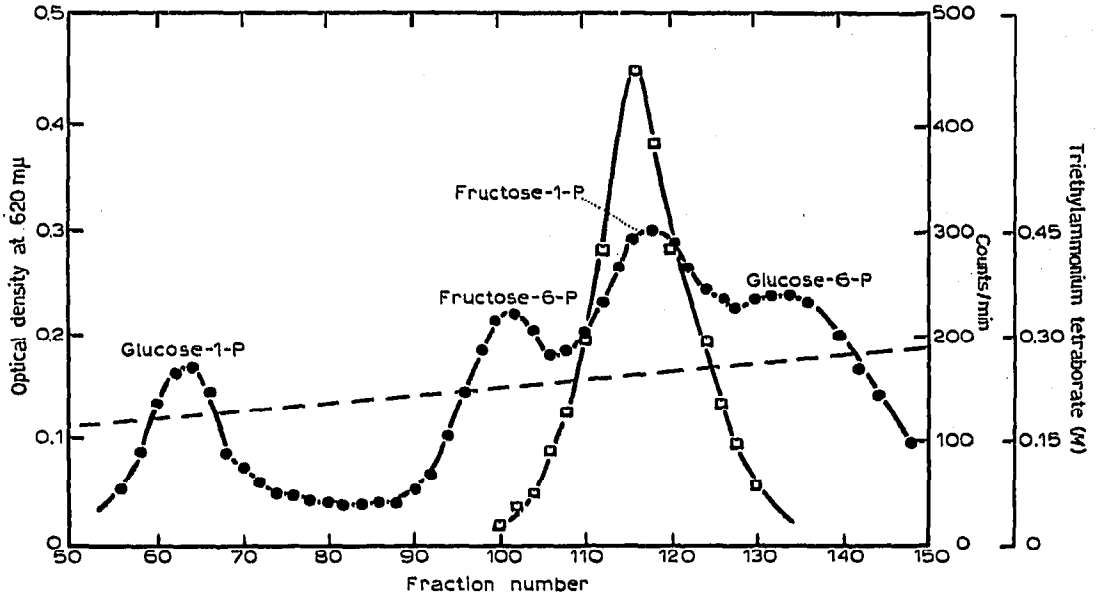

Fig. 3. Ion-exchange chromatography of fructose-1-P-^{14}C. The broken line represents the gradient from 0.1 *M* to 0.35 *M* triethylammonium tetraborate (300 ml total volume). Fructose-1-P-^{14}C 36,000 counts/min was mixed with a solution containing approximately 10 μmoles of each sugar phosphate and applied to the column. Counting of radioactive aliquots as described in text. ●, anthrone assay; □, counts/min.

As the free sugars are eluted at the beginning of the gradient (Fig. 1C), this method is very suitable for the purification of labelled sugar phosphates obtained through enzymatic reactions where adenosine triphosphate is the usual phosphorylating agent. This nucleotide, as well as adenosine mono- and diphosphate, is retained on the column.

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SUMMARY

A method is presented which allows the separation of most of the sugar phosphates on an anion-exchange resin column and permits their almost quantitative recovery. It is based on the use of linear gradient elution by ammonium or triethylammonium tetraborate and on the removal of these salts, after freeze-drying, by distillation with methanol.

REFERENCES

1 J. X. KHYM AND W. E. COHN, *J. Am. Chem. Soc.*, 75 (1953) 1153.
2 J. X. KHYM, D. G. DOBERTY AND W. E. COHN, *J. Am. Chem. Soc.*, 76 (1954) 5523.
3 M. GOODMAN, A. A. BENSON AND M. CALVIN, *J. Am. Chem. Soc.*, 77 (1955) 4257.
4 Y. HARA, *J. Biochem. (Japan)*, 46 (1959) 571.
5 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 459.
6 D. F. DIEDRICH AND L. ANDERSON, *Anal. Biochem.*, 2 (1961) 68.
7 A. L. RAYMOND AND P. A. LEVENE, *J. Biol. Chem.*, 83 (1929) 619.
8 H. G. PONTIS AND C. L. FISCHER, *Biochem. J.*, 89 (1963) 452.
9 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
10 W. TREVELYAN AND J. HARRISON, *Biochem. J.*, 50 (1952) 298.
11 J. H. ROE, *J. Biol. Chem.*, 107 (1934) 15.
12 J. L. REISSIG, J. L. STROMINGER AND L. F. LELOIR, *J. Biol. Chem.*, 217 (1955) 959.
13 W. D. KUMLER AND J. J. EILER, *J. Am. Chem. Soc.*, 65 (1943) 2355.
14 J. X. KHYM AND L. P. ZILL, *J. Am. Chem. Soc.*, 74 (1952) 2090.