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THE PURIFICATION OF PHOSPHATE ESTERS ON DEAE-CELLULOSE

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SUMMARY

The utility of DEAE-cellulose for separating phosphate esters is examined. Emphasis is given to the fractionation of labile derivatives which cannot readily be isolated by conventional methods. The factors involved in resolving these phosphate derivatives from contaminants such as inorganic phosphate, sulphate, chloride and free sugars on DEAE-cellulose are assessed and a procedure developed for their purification. The separation of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, glycerol-1-phosphate, fructose-1-phosphate and adenosine monophosphate on DEAE-cellulose is accomplished with methanolic solutions of ammonium carbonate and the order of elution is predicted.

INTRODUCTION

In studies on the isolation of sugar phosphates, either prepared chemically or extracted from natural sources, a variety of somewhat arbitrary methods is available for their fractionation which depends on such properties as the differential solubilities of the barium salts in water and alcohol–water mixtures[1], the ability of ion-exchange resins to effect their separation usually at acid pH (ref. 2) or else in the presence of borate[3], adsorption on charcoal[4] or on cellulose columns[5] under carefully controlled conditions. The diverse nature of these methods and the frequent lack of any obvious theoretical explanations for their utility tends to present a rather confusing picture when contemplating their respective advantages for the fractionation of novel phosphate esters. Invariably some preliminary investigations are required prior to their use in each case.

It became apparent from work carried out in this laboratory on the chemical synthesis of extremely acid-labile phosphate derivatives[6] such as fructofuranose-2-phosphate[7], deoxyribofuranose-1-phosphate[8] and isoprene phosphates[9], that an alternative approach to their isolation was essential if the chemical synthesis of these derivatives was to be accomplished satisfactorily. The susceptibility of these compounds to hydrolysis in the presence of calcium and barium ions[10] and the inability to react with borate due to the lack of neighbouring *cis*-hydroxyl groups underline the weakness of the existing methods for their isolation. As the instability of these esters precluded the use of conventional methods for their separation, recourse was made to the use of a DEAE-cellulose column. The choice of this

material was encouraged primarily by a report of RUSHIZKY AND SOBER[11] that mono- and oligonucleotides could be desalted by the carbonate form of this exchanger if the mixture to be desalted was applied to the column in highly dilute ammonium carbonate at pH 8.6. They had observed that chloride and sulphate present in the nucleotide solutions emerged from the columns immediately under these conditions but that phosphates were delayed somewhat and that nucleotides required a change of eluting conditions. Thus it was hoped that the slight retentive behaviour of the exchanger towards phosphate could be turned to advantage in the present instance. The fact that a mildly alkaline environment was maintained throughout should enable the acid-labile esters to be isolated with a minimum of hydrolysis.

The work described here is the culmination of preliminary studies on the efficacy of DEAE-cellulose for separating phosphate esters from the usual contaminants, *viz.* sugar, chloride, sulphate and inorganic phosphate present in a reaction mixture for the chemical synthesis of sugar phosphates. The retention behaviour of these constituents is discussed in some detail, explanations are given where possible for the separations obtained, and an attempt is made to utilise this DEAE-cellulose system to resolve phosphate ester mixtures into individual species in a predictable manner.

MATERIALS AND METHODS

Materials

DEAE-cellulose of 1.0 mequiv./g nominal capacity and cellulose powder ("microcrystalline grade") for thin-layer chromatography, manufactured by W. and R. Balston Ltd., Great Britain, were used throughout. The samples of G-1-P, G-6-P, F-6-P, F-1-P, glycerol-1-P, AMP, glucose, ammonium sulphate, ammonium chloride and ammonium carbonate were purchased from B.D.H. Chemicals Ltd., Great Britain.

Methods

DEAE-cellulose was suspended in a large volume of water and after a brief settling period the supernatant was decanted. This procedure was repeated several times until all the fines were removed. The residue was made into a fine slurry with 1 *M* ammonium carbonate solution and poured with stirring into a column already half filled with the same solution. This approach was found to produce the most uniform column packing with a minimum of channeling. The DEAE-cellulose column of dimensions 1.8 cm diameter by 15 cm thus obtained was compacted slightly and used in this form after equilibrating with 0.01 *M* ammonium carbonate either in aqueous or in 70 % aqueous methanol solution as required. To obtain satisfactory flow rates of ~1 ml/min the columns had to be repacked for each new sequence of events.

Amounts of 10 mg of each of the compounds $(NH_4)_2SO_4$, NH_4Cl, glucose and various permutations of G-1-P, G-6-P, F-6-P, F-1-P, glycerol-1-P, and AMP as described in the text were made up to 100 ml with either aqueous or 70 % aqueous methanolic solutions of 0.01 *M* ammonium carbonate and applied to the DEAE-cellulose. The columns were then washed with these solutions until the effluents were

chloride-free, after which solutions of increasing ammonium carbonate concentration were applied to remove the components held on the substituted cellulose.

The elutions of chloride and sulphate were followed visually by the addition of acidified silver nitrate and barium chloride, respectively. The phosphate esters were analysed by the method of AMES AND DUBIN[12], total sugar by phenol–sulphuric acid[13] and fructose by the procedure of ROE *et al.*[14].

The thin-layer plates were developed with 0.01 *M* ammonium carbonate in 70% aqueous methanol as solvent and the sugars located with an alkaline silver nitrate reagent[15] and phosphate esters with acid molybdate[16].

RESULTS AND DISCUSSION

In the separation of phosphate esters from free sugar, chloride and sulphate contaminants on DEAE-cellulose with ammonium carbonate solutions, the order of elution can be predicted solely on the basis of charge. At pH 8.6 the charges on the relevant anions are: chloride, −1; sulphate, −2; phosphate esters, −2; and bicarbonate, −1. The latter is the actual species present at pH 8.6 and not carbonate, as suggested in the text (pK of $HCO_3^- \rightleftharpoons CO_3^{2-}$, 10.6). Therefore the phosphate esters and sulphate should be retained on the exchanger at the expense of chloride and bicarbonate. This was confirmed in all examples tested and is illustrated in Fig. 1 with G-1-P as the phosphorylated species. The free sugar is of course not retained, chloride is retarded slightly, while G-1-P is held on the column and will ultimately be eluted with this solvent although it is customary to encourage its removal by applying a higher concentration of ammonium carbonate. Sulphate is firmly held on the exchanger and requires a high salt concentration for its release. The retention behaviour of sulphate, illustrated here, which is typical of all samples

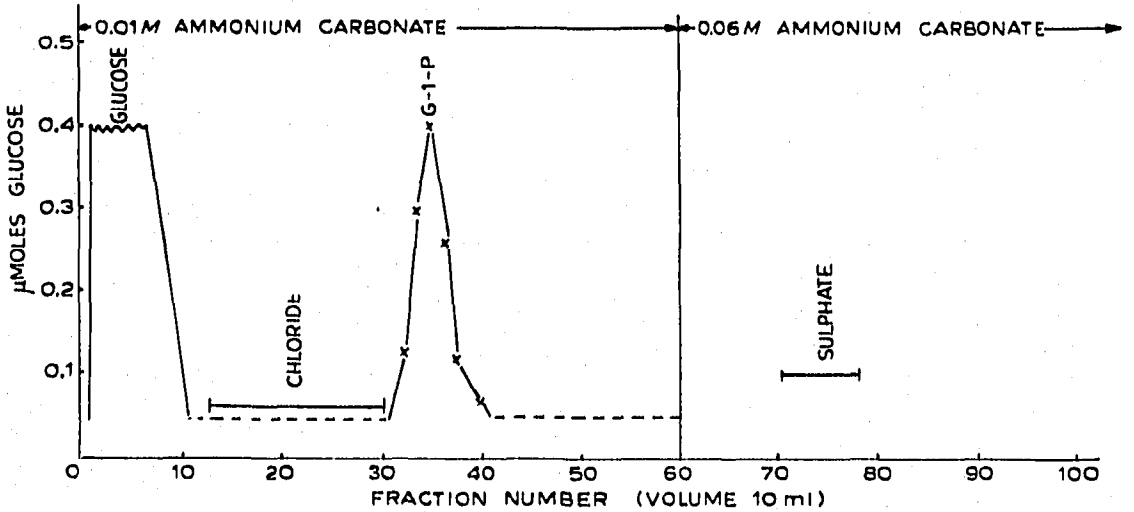

Fig. 1. Elution sequence for glucose, G-1-P $(NH_4)_2$, NH_4Cl, and $(NH_4)_2SO_4$ on a DEAE-cellulose column (1.8 cm diameter × 15 cm), using aqueous solutions of ammonium carbonate. Amounts of 10 mg of each component were dissolved in 0.01 *M* ammonium carbonate, combined, adjusted to 100 ml and applied directly to the DEAE-cellulose. Progress of elution was followed by estimating the sugar concentration (expressed as glucose) of 0.5-ml aliquots of column effluent with phenol–sulphuric acid[13]. Phosphate, chloride and sulphate were assayed as described in the text.

studied, does not agree with the reported figure of RUSHIZKY AND SOBER[11], who claim that sulphate together with chloride is not retained on DEAE-cellulose in the presence of 0.01 *M* ammonium carbonate. Nevertheless in spite of the anomalous behaviour of sulphate in these two systems, G-1-P and a variety of other phosphate esters can be obtained free of chloride and sulphate using the DEAE-cellulose system here described. By bulking appropriate fractions and evaporating the solution under vacuum the ammonium carbonate slowly evaporates off leaving in this case the diammonium salt of G-1-P as a deliquescent white powder. By similar means a selection of chemically synthesised sugar phosphates including fructofuranose-2-phosphate[6] was isolated in excellent yields, free of extraneous salts. To accomplish this the inorganic phosphate invariably present must first be removed as insoluble magnesium ammonium phosphate by the addition of magnesia mixture, before submitting the sample to the DEAE-cellulose procedure. The alkaline conditions maintained throughout enable these acid-labile derivatives to be submitted to this procedure with the utmost confidence.

It was noted, however, that in the presence of very high salt concentrations, when substantial dilutions were required before the DEAE-cellulose treatment, flat peaks frequently occurred with resulting contamination between fractions. To minimise these effects due to high salt levels it was found expedient to carry out the chromatography in aqueous methanol rather than in totally aqueous solution. This enabled a better separation of the components present to be attained, presumably by encouraging the participation of other processes such as partition and adsorption effects, to influence the separation on DEAE-cellulose, attributed above, mainly in aqueous solution, to ion exchange. Similar reasoning has been applied to the separation of complex lipid mixtures on this exchanger with marked success[17,18].

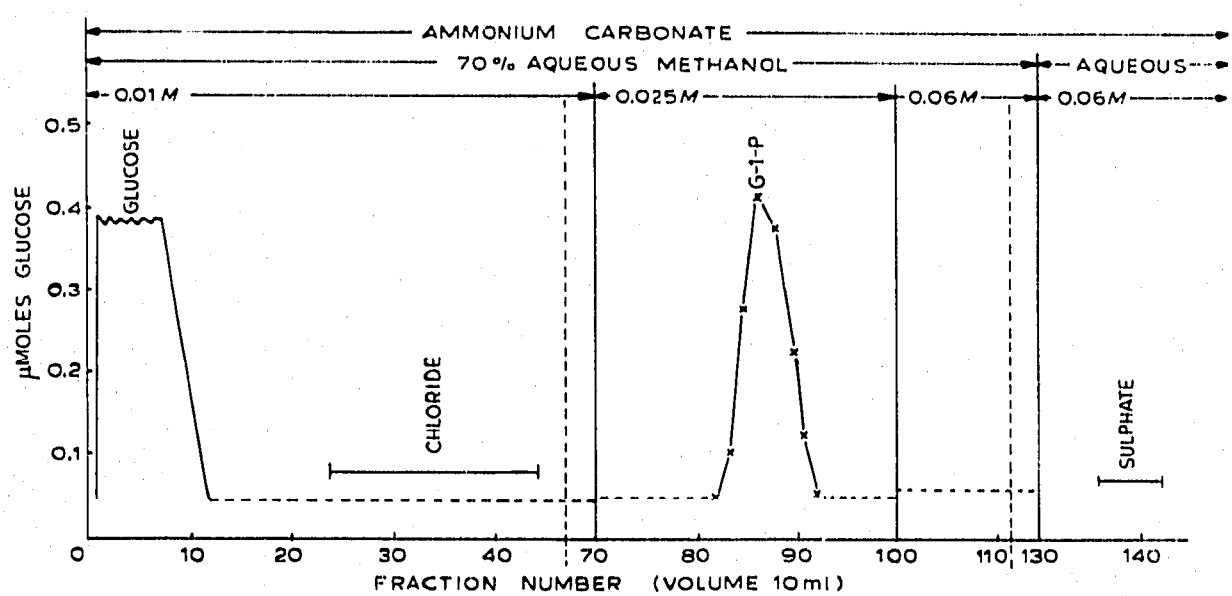

Fig. 2. Elution sequence for glucose, G-1-P $(NH_4)_2$, NH_4Cl and $(NH_4)_2SO_4$ on a DEAE-cellulose column (1.8 cm diameter × 15 cm), using 70% aqueous methanol solutions of ammonium carbonate. Amounts of 10 mg of each component were dissolved in a 70% aqueous methanol solution of 0.01 *M* ammonium carbonate, combined, adjusted to 100 ml and applied directly to the DEAE-cellulose. Progress of elution was followed as for Fig. 1.

After some preliminary work 70 % aqueous methanol was selected as suitable for separating phosphate esters from high concentrations of chloride and sulphate. Under comparable conditions to these illustrated above for G-1-P except for the inclusion of 70 % aqueous methanol in the solvent (Fig. 2), sugar was unaffected, chloride retention was slightly enhanced, G-1-P elution required a substantial increase in salt concentration and sulphate, in turn, was firmly held, recourse having to be made to aqueous solutions for its removal. In this system, contamination between fractions was remote, as G-1-P was eluted well, clear of both chloride and sulphate. Even when the contaminant levels were increased by the order of ten, no difficulty was experienced in obtaining pure samples of G-1-P, although the retention behaviours of individual phosphate esters were found to vary as would be expected on occasion, due to differences in acidities[19]. In any of the examples studied problems of contamination have not arisen while using this latter variation.

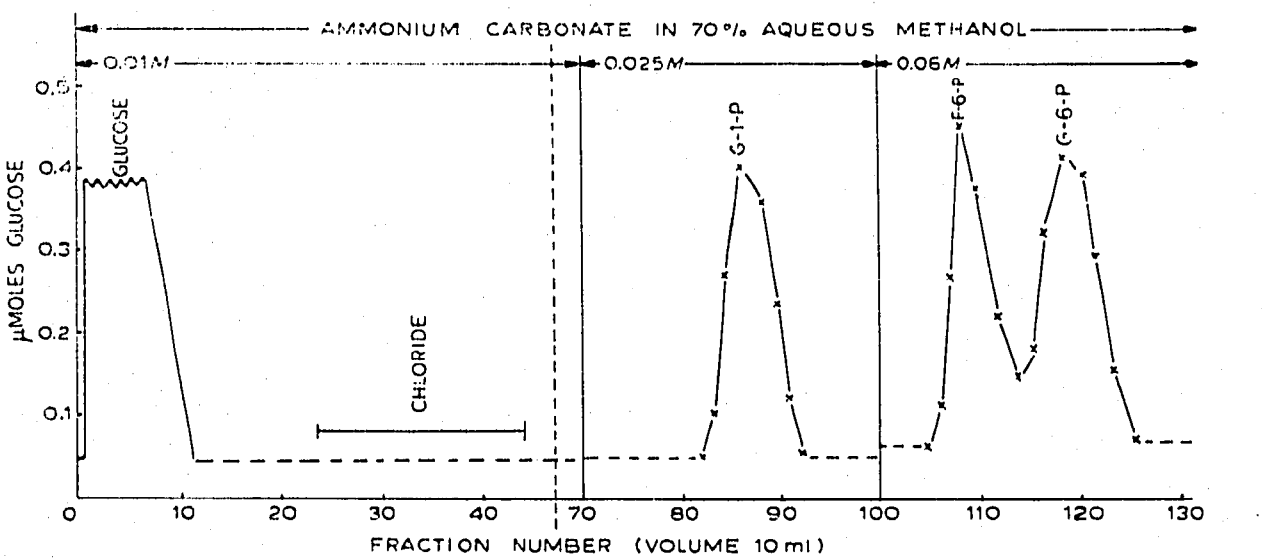

Fig. 3. Elution sequence for glucose, NH_4Cl, G-1-P$(NH_4)_2$, G-6-P$(NH_4)_2$ and F-6-P$(NH_4)_2$ on a DEAE-cellulose column (1.8 cm diameter × 15 cm) using 70% aqueous methanol solutions of ammonium carbonate. Amounts of 10 mg of each component were dissolved in a 70% aqueous methanol solution of 0.01 *M* ammonium carbonate, combined, adjusted to 100 ml and applied directly to the DEAE-cellulose. Progress of elution was followed as for Fig. 1. In addition, the presence of fructose was confirmed by the method of Roe *et al.*[13].

In an attempt to utilise these differences in retention behaviours of phosphate esters on DEAE-cellulose, G-1-P, G-6-P and F-6-P (sugar phosphates of comparable acidities) were bulked and submitted to the DEAE-cellulose treatment using the aqueous methanol solutions. Under these conditions resolution of the three phosphorylated sugars was obtained (Fig. 3), the order of elution being G-1-P, F-6-P and G-6-P. In this instance the differences in retention behaviour between the phosphate esters cannot be accounted for solely on the basis of ion exchange; other factors such as interactions with the cellulose matrix must be involved in influencing the separation[20]. To investigate this point further the three sugar phosphates were chromatographed on thin-layer plates coated with cellulose and developed with 0.01 *M* ammonium carbonate in 70 % aqueous methanol. The R_P values obtained for G-1-P, F-6-P

and G-6-P (0.80, 0.65 and 0.60, respectively) tend to indicate in this situation that where the acids are of comparable strengths, the resolution on the DEAE-cellulose can be accounted for on the basis of cellulose chromatographic data, the faster moving components being more readily released by the exchanger. It is also worthy of note that the interactions between the cellulose matrix and the anions present will be enhanced in this situation due to the electrostatic forces involved.

An identical experiment to the above with glycerol-1-P, AMP and F-1-P (Fig. 4) again illustrates the feasibility of separating phosphate esters on DEAE-cellulose with aqueous methanolic solutions of ammonium carbonate.

Clearly before commenting more fully on the utility of this procedure for separating phosphorylated species into individual components further studies are required. Increased column length and the incorporation of a gradient elution system instead of the batch procedure utilised here would be two obvious refinements which should lead to better resolutions. However, at this juncture, it suffices to say that in the case of G-1-P, F-6-P, G-6-P, F-1-P, glycerol-1-P and AMP, compounds differing only minimally in acid strength, separations can be effected on DEAE-cellulose in a predictable manner using 70 % aqueous methanol solutions of ammonium carbonate.

Under the conditions specified the order of elution and the corresponding fraction numbers of these phosphate derivatives are G-1-P (80–90), glycerol-1-P (94–101), F-1-P (104–109), F-6-P (108–112), G-6-P (113–119) and AMP (122–130).

With this DEAE-cellulose column the retention behaviours of chemically synthesised samples of fructofuranose-2-phosphate[6] (88–94) and fructopyranose-2-phosphate[6] (105–111) are as predicted by their R_F values (0.75 and 0.65, respectively),

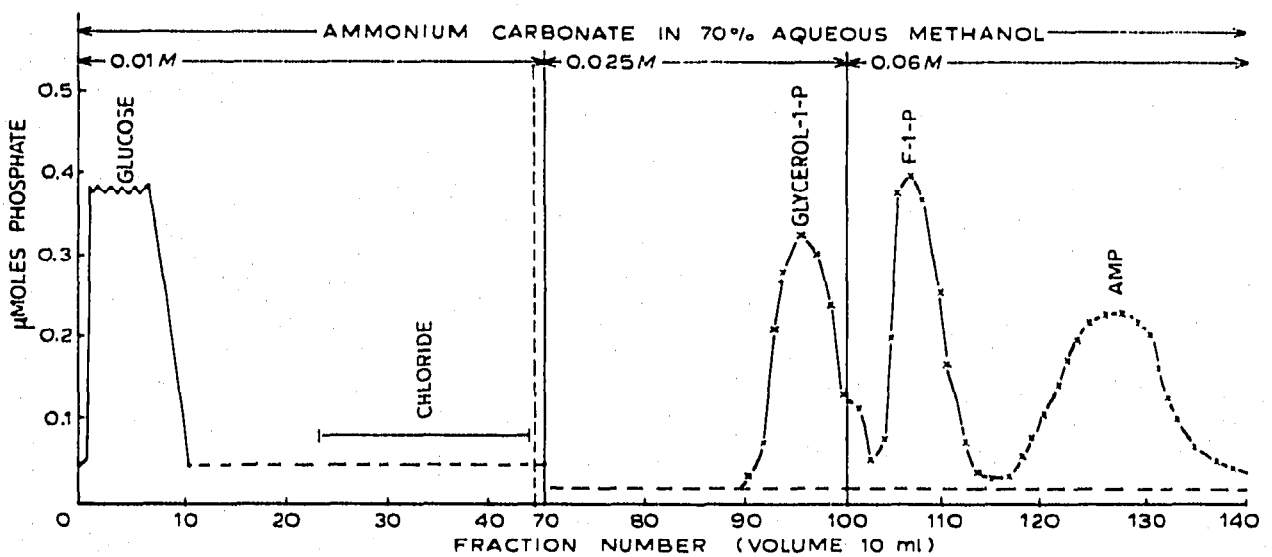

Fig. 4. Elution sequence for glucose, NH_4Cl, glycerol-1-P$(NH_4)_2$, F-1-P$(NH_4)_2$ and AMP$(NH_4)_2$ on a DEAE-cellulose column (1.8 cm diameter × 15 cm) using 70% aqueous methanol solutions of ammonium carbonate. Amounts of 10 mg of each component were dissolved in a 70% aqueous solution of 0.01 *M* ammonium carbonate, combined, adjusted to 100 ml and applied directly to the DEAE-cellulose. Progress of elution was followed by estimating the phosphate content of 0.5-ml aliquots of column effluent, by the method of AMES AND DUBIN[12]. The presence of AMP was confirmed by E_{260} measurement, and chloride and fructose were assayed as described in the text.

using the above solvent, the former resembling G-1-P and the latter F-1-P under these conditions.

Finally it should be added that aqueous methanol solutions have the additional merit, when isolating chemically synthesised samples of sugar phosphates, of reducing the number of steps involved. At the deacetylation stage customarily carried out in 70 % aqueous methanol and after filtering off the insoluble trisodium phosphate, the clear supernatant is applied directly to the DEAE-cellulose column thus eliminating the need for solvent evaporation and resolution in large volumes of 0.01 *M* ammonium carbonate prior to the column treatment. The sugar phosphate is of course recovered in high yield, free of contaminants as earlier described.

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