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CHROMATOGRAPHIC SEPARATION OF BIOLOGICALLY IMPORTANT PHOSPHATE ESTERS*

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SUMMARY

A column chromatographic procedure formerly applied to separations of sugar phosphates on Dowex 1 chloride has been used in resolving a variety of nucleotides and coenzymes. Group separations have been effected by this method. For further separation and purification of common nucleotides, a linear gradient procedure has been presented. In this system, Dowex 1 chloride or formate columns are used with either hydrochloric acid, formic acid, ammonium formate, or ammonium chloride as eluants.

INTRODUCTION

The separation of complex mixtures of phosphate esters such as nucleotides and coenzymes by column chromatography has received much attention[1-8]. Recently, emphasis has been placed upon rapid analyses which are characterized by column systems with relatively high operating temperatures[1,2,4] and, in one case, with very high pressure[2]. Such systems achieve excellent separations of the common nucleotides but do have several inherent disadvantages. For example, HORVATH *et al.*[2] used a pellicular ion exchanger at 71° and 51 atm. This system handles only nanomole quantities of each component, making preparative scale work, and hence identification of unknown compounds, difficult. Furthermore, high temperature, despite a short operating time, presumably causes decomposition of labile phosphate bonds. In this regard, PLAISTED AND REGGIO[1], using Dowex 1 formate** at 37°, found up to 15% loss of mg samples of nucleoside triphosphates in 17 h. For this reason we chose to operate our column systems at room temperature (25°).

Single column systems additionally have disadvantages if they are to be used

* The following abbreviations will be used: P = phosphate; CMP, AMP, GMP, TMP, UMP = cytidine-, adenosine-, guanosine-, thymidine-, and uridine-5'-monophosphates; CDP, ADP, GDP, TDP, UDP = the corresponding diphosphates; CTP, ATP, GTP, UTP = the corresponding triphosphates; NAD = nicotinamide adenine dinucleotide; NADP = nicotinamide adenine dinucleotide phosphate; FMN = flavin mononucleotide; FAD = flavin adenine dinucleotide.

** Mention of trade or company names does not imply endorsement by the Department over others not named.

in analyzing nucleotides present in acid-soluble extracts of tissues. Such extracts usually contain a very complex mixture of compounds which, according to HURLBERT *et al.*[8], cannot be completely separated in any single column system. They suggest the use of a second system that can provide a different distribution coefficient for a given compound. Other investigators found that numerous anions, such as would be present in plant extracts, can compete with phosphates for exchange sites on the resin, thereby changing retention volumes and destroying resolution[1]. PONTIS *et al.*[3] observed continuous tailing of nucleotides and diminished column capacity which they attributed to large amounts of riboflavin and other pigments in an *E. ashbyii* extract. These problems were overcome by rechromatographing the extract on a second Dowex 1 column[3]. Finally, LIN AND HASSID[9] discovered that, whereas subfractionation of nucleotides in a second column system only slightly improved separation of individual components, it did, nevertheless, remove ultraviolet-absorbing contaminants.

BARTLETT[10] described a procedure for rapidly separating glycolytic sugar phosphates on Dowex 1 chloride with a stepwise increase in chloride concentration. In this paper, we have extended the analyses in BARTLETT's system to include common nucleotides and coenzymes which may be found in biological extracts. Secondly, we wish to present an elution schedule for the further purification and separation of nucleotides not well resolved in the BARTLETT system.

EXPERIMENTAL

Materials

Glucose-1-P, glucose-6-P, TMP, TDP, and CDP were purchased from Calbiochem. All other phosphates were obtained from Sigma Chemical Co. Purity of the test compounds was verified by noting their movements as single components on TLC plates in one or more of the following systems: (a) *n*-propanol–25% NH_3–H_2O (6:3:1)[11], (b) methanol–aqueous NH_3–H_2O (7:1:2)[12], and (c) tertiary amyl alcohol–formic acid–H_2O (3:2:1)[13].

Analytical procedures

FMN, FAD, and pyridoxal phosphate were monitored at 355 mμ, while other phosphates were monitored at 260 mμ in a Beckman DU spectrophotometer. In general when mixtures were applied to a column, the 280/260 and 290/260 absorbancy ratios were used for identification[14]. Sugar phosphates were determined by the anthrone method[15].

Column chromatography

Bartlett system. Dowex 1 X8 anion exchange resin, 50–100 mesh, was allowed to sediment in distilled water, and the fines were removed by decantation. The resin was then poured as a slurry into a 1 × 14 cm column and converted to the chloride form with 1 *N* HCl (300 ml). Distilled water was finally passed through the resin until the pH of the effluent increased to six. Phosphates were applied to the column as either their sodium or ammonium salts, singly, or in mixtures, in amounts between 4 and 40 mg in about one ml of distilled water (pH 7). Eluate was collected at a rate of 2 to 3 ml per min under conditions of atmospheric pressure and room temperature (25°).

TABLE I

SEPARATION IN BARTLETT'S SYSTEM

Compound	*Eluant*	*Retention volume (ml)*[a]
Vitamin B_{12}	0.003 *N* HCl	Present in void volume
CMP		20
NAD		30
AMP[b]		80
Pyridoxal-P		180
Adenosine-2',3'-cyclic-P	0.01 *N* HCl	310
NADP		320
Glucose-6-P[b]		320
Glucose-1-P		
Fructose-1-P		
Fructose-6-P[b]		
GMP		370
TMP		390
UMP		390
CDP		410
ADP-glucose	0.02 *N* HCl	610
ADP[b]		620
FMN		620
Coenzyme A		630
FAD		670
UDP	0.1 *M* NH_4Cl	940
TDP		1030
GDP	0.2 *M* NH_4Cl	1220
CTP		1350
UTP		1350
GTP	0.5 *M* NH_4Cl	1510
ATP[b]		1530

[a] Volume of eluate to peak concentration.
[b] Materials previously reported by BARTLETT and chromatographed on our column for reference purposes.

Elution was carried out with 300 ml portions of the eluants used in BARTLETT's procedure[10] (see Table I).

Linear gradient system. Dowex 1 X8 resin, 200–400 mesh, was pre-treated in the manner given above and then poured into two 1 × 28 cm columns. One column was converted to the formate form with 200 ml of 5 *M* sodium formate, the other to the chloride form. Common nucleotides which separated poorly or not at all in BARTLETT's system were resolved by rechromatography using chloride or formate linear gradients in a total volume of two liters. Flow rates averaged about 0.5 ml per min.

RESULTS AND DISCUSSION

Bartlett system

A variety of nucleotides, coenzymes, and sugar phosphates were applied as standards to BARTLETT's system. Previously retention volumes had been reported

only for carbohydrate phosphates, AMP, ADP, and ATP by this procedure[16]. From Table I it can be seen that this method effects group separations. In general, there is a tendency for compounds with the greatest net negative charge to be eluted last as the chloride ion concentration increases. Another characteristic of the system is tailing peaks which are obtained when certain nucleotides or coenzymes are chromatographed. On the other hand, the carbohydrate phosphates analyzed were sharply removed despite some peak "heading". Although many compounds do not separate in this system, nevertheless, they can be determined either by monitoring at a wavelength other than 260 mμ, or they can be rechromatographed in a second column system. Thus pyridoxal phosphate, which partly overlapped AMP, can be determined in its presence if read at 355 mμ. Likewise, FAD and FMN absorb at 355 mμ. They can be quite satisfactorily resolved by rechromatography on a short cellulose column[17]. The four sugar phosphates also can be rechromatographed with complete separation on Dowex 1 borate[18].

Vitamin B_{12} is the only compound that we have tested which appeared in the void volume. Its exclusion may have been the result of either its large molecular size or a net charge of zero. (In this compound the phosphate can theoretically be situated in close proximity to the cobalt.) The order of elution of CMP and AMP on Dowex 1 (by acid) has been explained by COHN[6] on the basis of amino and phosphate group pK values. Accordingly, as the pH of the eluant in the beads falls, CMP approaches a net charge of zero in advance of AMP. Similarly NAD eluted early, because of the positive charge on its pyridine nitrogen, while pyridoxal phosphate was held longer than those already mentioned, because it does not have a cationic group. Furthermore, in evaluating the importance of the phosphate dissociations in two forms of AMP, the 5′- and and the 2′, 3′-cyclic isomers, it is observed, but without explanation, that the latter substance was held more tenaciously to the resin than was the former (Table I). In this connection it is noted that the 5′- isomer has two dissociations, the cyclic isomer, one. Meanwhile, ADP with three phosphate ionizations eluted with ADP-glucose, which has but two. In this case, however, the strong eluting force of 0.02 N HCl may have been a factor in masking the effect of the number of phosphate ionizations on retention volume. In contrast, the sugar phosphates probably eluted together because of similar primary and secondary pK values[19].

As a generality, one can state that nucleoside phosphates of cytosine are removed from the resin first in each series of ribonucleoside mono-, di-, and triphosphates.

Linear gradient system

For resolution of compounds with similar partition properties for a given exchanger and eluant, it is often helpful to increase the length of the column or to decrease bead size, or both, thereby increasing the number of exchange sites[1]. This modification, in effect, increases the number of "operations" in the separation process. For greater resolution in the linear gradient system over that in BARTLETT's procedure, we chose to use a relatively long column (28 cm) with a 200–400 mesh resin, and, in several cases, formate eluants based upon those employed by HURLBERT *et al.*[8]. In this regard, formic acid and its ammonium salt are advantageous eluting agents, because they provide good pH control and can be easily removed from the sample by evaporation[20].

In Fig. 1 it can be seen that CMP and AMP, which eluted from the 0.003 N HCl section of BARTLETT's system with some overlap, could be completely separated in a

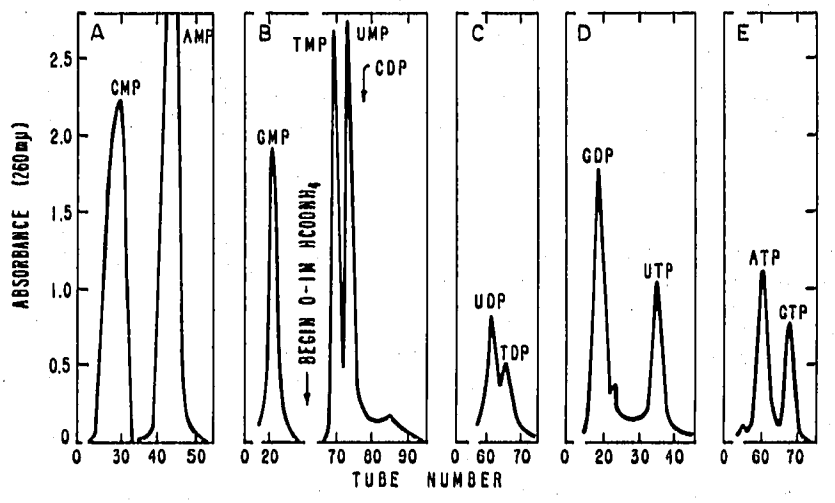

Fig. 1. Separation of nucleotides by linear gradients. All columns were 1 × 28 cm, containing 200–400 mesh particles with 8% crosslinkage. Standard nucleotides were applied in amounts between 3 and 10 mg. Eluates were collected, 20 ml/tube. (A) Dowex 1 chloride, 0 to 0.009 *N* HCl; (B) Dowex 1 formate, 600 ml of 1 *N* HCOOH to 3 *N* HCOOH, followed by 0 to 1 *M* $HCOONH_4$; (C) Dowex 1 chloride, 0 to 0.15 *M* NH_4Cl; (D) Dowex 1 formate, 4 *N* HCOOH to 1 *M* $HCOONH_4$ + 4 *N* HCOOH; (E) Dowex 1 formate, 0.2 *M* $HCOONH_4$ to 1 *M* $HCOONH_4$. Operating conditions: room temperature and atmospheric pressure with an average flow rate of 0.5 ml per min.

gradient of 0–0.009 *N* HCl. At this concentration, HCl can be effectively removed from the samples by freeze-drying. GMP, UMP, TMP, and CDP were rechromatographed in the following way. Six hundred milliliters of a gradient of 1 *N* HCOOH to 3 *N* HCOOH was run (enough volume to elute GMP), followed by a change to a 0 to 1 *M* $HCOONH_4$ gradient. The structurally similar UMP and TMP could be readily distinguished by this method, although resolution of CDP and UMP is lost (Fig. 1). ADP was the only common mononucleotide present in the 0.02 *N* HCl section and was not applied to a gradient system. UDP and TDP could be more sharply separated in a gradient of 0 to 0.15 *M* NH_4Cl. This eluant, according to BARTLETT[10], is particularly mild, allowing one to keep samples at 5° for weeks. Likewise, GDP was resolved more completely from UTP when chromatographed in 4 *N* HCOOH to 1 *M* $HCOONH_4$ + 4 *N* HCOOH. And lastly, ATP and GTP, which were removed in one peak with 0.5 *M* NH_4Cl, were separable in a gradient of 0.2 *M* $HCOONH_4$ to 1 *M* $HCOONH_4$.

In general, it may be noted that all peaks in Fig. 1 are very sharp and symmetrical indicating the high degree of efficiency obtainable with this system of eluting agents. Admittedly, the total volume of eluant used is large, but the procedure can be speeded considerably by running several gradients, *viz.* A, B, C, etc., simultaneously. When this is done, total elution time for this procedure becomes comparable with single column systems[4,5,7].

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