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RAPID SEPARATION OF NUCLEOSIDES AND NUCLEOTIDES BY CATION-EXCHANGE COLUMN CHROMATOGRAPHY

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SUMMARY

Three methods for separation of nucleosides and nucleotides on the cation exchanger AG 50W–X4, at alkaline pH, are described. Two of the methods, one for the deoxy-series and one for the ribo-series, separate the nucleotides together and the nucleosides into the individual components. Regeneration of the columns is not required for re-use. The third method separates completely all nucleotides and nucleosides. All separations are complete in one and a half to two hours.

INTRODUCTION

Methods used for the determination of the sequence of oligonucleotides include digestion by various enzymes and separation, identification and quantitation of the products. Following removal of terminal phosphate groups the enzyme used is usually an exonuclease such as snake venom phosphodiesterase (EC 3.1.4.1.) which releases the terminal bases as nucleosides and the internal bases as nucleotides[1–3]. The large number of oligonucleotides released by partial degradation of even the smallest nucleic acids[4] necessitates that the methods of analysis for sequence be both rapid and quantitative. Recently a number of methods for separation of nucleosides or nucleotides have been reported[5–9]. Two of these methods[5,8] are rapid, that is, can be accomplished in 1 to 1.5 h. The method described by BLATTNER AND ERICKSON[5] is applicable only to nucleotides and that of UZIEL *et al.*[8], for nucleosides, has been developed for use with a highly automated chromatographic system and will not separate nucleotides from nucleosides under the conditions described. We have designed procedures for both the ribo- and deoxyribo-series in which a mixture of nucleotides and nucleosides can be separated by column chromatography within 1.5 to 2 h. The procedures have the additional advantages that the columns do not need regenerating but can be used many times, the sample material can contain enzymes and buffer salts and the eluting solvents are volatile.

EXPERIMENTAL

The cation exchanger used is AG 50W-X4, minus 400 mesh (Calbiochem, Los Angeles, Calif., U.S.A.). The resin was washed twice with 10 vol. of 1 *N* HCl,

three times with 10 vol. of concentrated NH_4OH followed by three washes with 10 vol. of the elution buffer to be used[5]. Fines were removed by decantation.

Nucleosides and nucleotides were purchased from Calbiochem and enzymes from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. *E. coli* alkaline phosphatase (EC 3.1.3.1.) was assayed with sodium *p*-nitrophenylphosphate (British Drug Houses Ltd.) and snake venom phosphodiesterase (EC 3.1.4.1.) with calcium bis-*p*-nitrophenylphosphate (Sigma Chemical Co.) Bovine spleen phosphodiesterase (EC 3.1.4.1.) was not assayed prior to use since it was used in excess.

Column preparation and elution

Chromatography columns were glass, 11 mm I.D. with internally threaded ends (Ace Glass Incorporated, Vineland, N.J., U.S.A.). They were fitted with a polyethylene filter disc, pore size 100 μ, and a nylon bottom drip adapter with a Luer fitting which connected to Chromatronix (York Instrument Company, New York, N.Y., U.S.A.) teflon tubing adaptors. These column end fittings eliminated dead volume at the end of the column so that mixing of the eluted material was minimized. Columns were packed with a 50% slurry of the resin and washed with the corresponding elution buffer for 1 h prior to use. Columns stored for even a few hours were routinely washed 1 h before use. Flow rate was maintained with the use of a Buchler polystaltic pump at 70–100 ml/h. The internal threading of the glass columns was required to withstand the back pressure generated by the pump. All columns were operated at room temperature. Sample volumes ranged from 0.02 to 2.2 ml. Enzyme digestion mixtures were adjusted to the pH of the elution buffer and loaded directly on the column. The sample was allowed to soak completely into the resin bed then the column eluted immediately. The UV absorption of the column effluent was determined with a Gilford 2000 spectrophotometer at 260 mμ, or an Isco model UA-2 UV monitoring system at 254 mμ. Two milliliter fractions were collected.

Sample preparation

Commercial nucleosides and nucleotides of the ribo- and deoxyribo-series were dissolved in the appropriate chromatography buffer to give a final solution of 0.5–0.8 A_{260} units or 5–17 A_{260} units per nucleotide or nucleoside.

Deoxyoligonucleotides obtained from DNase 1 digests were fractionated according to chain length[10] and the fractions digested with 0.5 units of *E. coli* alkaline phosphatase per 30–100 A_{260} units dissolved in 0.05 *M* Tris–HCl buffer pH 8.0, for 6 h at 37°, total volume 2.0 ml. Phosphatase was removed by four phenol extractions followed by four ethyl ether extractions to remove the excess phenol. The ether was removed by evaporation in a water bath at 50° for 15 min. 1.5 ml of the phosphatase treated material was adjusted to pH 8.8 with 0.1 *M* Trizma base (Sigma Chemical Company, St. Louis, Mo., U.S.A.), pH unadjusted, containing 0.03 *M* $MgCl_2$, and 0.2 units of snake venom phosphodiesterase were added and the mixture incubated for 12 h at 37°. A second 1.5 ml sample of the phosphatase treated material was mixed with 0.4 ml of 0.5 *M* sodium acetate buffer pH 6.5 and to this 1 unit of spleen phosphodiesterase was added and the mixture incubated for 24 h at 37°. Following adjustment of the pH for chromatography, as described above, these samples were applied directly to the chromatographic columns.

Chromatographic systems

Three different chromatographic elution conditions have been developed.

Separation of 3'- or 5'-deoxynucleotides and deoxynucleosides. Column, 42 cm by 11 mm; elution buffer, ammonium formate prepared by adjustment of 0.3 *M* NH_4OH to pH 9.2 with concentrated formic acid.

Separation of 3'- or 5'-ribonucleotides and ribonucleosides. Column, 60 cm by 11 mm, buffer, ammonium formate prepared by adjustment of 0.3 *M* ammonium hydroxide to pH 8.9 with concentrated formic acid.

Fractionation of all four deoxynucleosides and 5' deoxynucleotides. Column, 42 cm by 11 mm; buffer, ammonium formate prepared by adjustment of 0.015 *M* ammonium hydroxide to pH 3.2 with formic acid. Following elution of the first five components with this buffer a second ammonium formate buffer, prepared by adjustment of 0.3 *M* ammonium hydroxide to pH 9.2 with formic acid, is applied.

A Radiometer, Model TTT 1c pH meter, calibrated at 20° and standardized at pH 4, 7 or 10 daily, was used for all pH measurements.

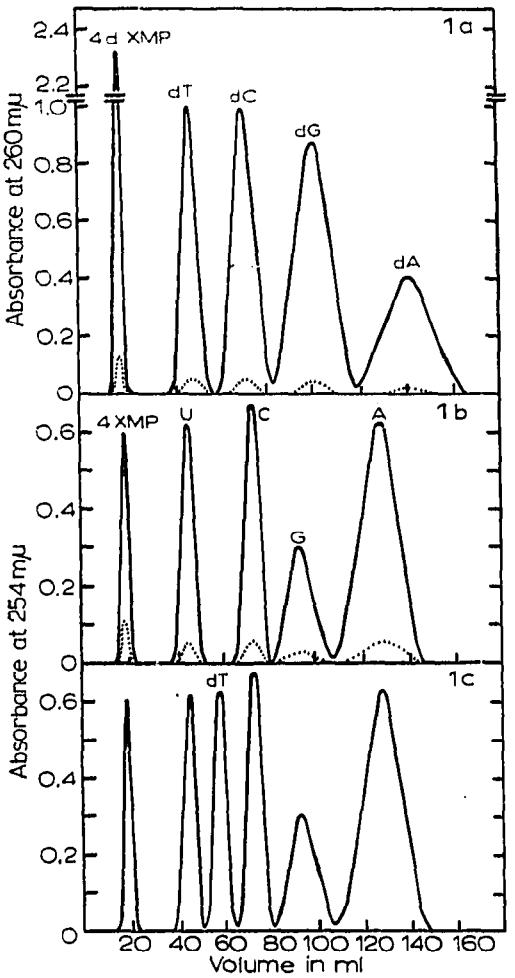
All buffers must be freshly prepared since being volatile they are susceptible to pH change upon standing. The elution buffers are the same as those used for loading the columns and the first two columns automatically regenerate themselves with elution and no further regeneration is required. Nucleotides and nucleosides were distinguished by phosphate analysis and the separated components identified by spectral analysis.

RESULTS

Separation of 3'- or 5'-deoxynucleotides and deoxynucleosides and 3'- or 5'-ribonucleotides and ribonucleosides

The chromatographic separation of two mixtures of 5'-deoxynucleotides and deoxynucleosides is shown in Fig. 1a and two mixtures of 5'-ribonucleotides and ribonucleosides in Fig. 1b. Both sets of mixtures contained identical components in equal proportions but differed in concentration by a factor of 20 for the deoxy compounds, and 12 for the ribo compounds. All four nucleotides were eluted together in these systems and the four nucleosides then separated into the individual components in the elution order thymidine or uridine, cytidine, guanosine and adenosine. The separated components were analyzed quantitatively by pooling the peaks, adjustment of the pH and spectral analysis. The results presented in Table I list both recoveries and the volume of the fractions eluted from the column for the largest sample. The results shown in Fig. 1a and b and Table I are those obtained by the optimal conditions listed under EXPERIMENTAL. Variations in column length, column diameter, flow rate, molarity and pH of the eluting buffer were all investigated. The separation is extremely pH dependent. Variations of as little as 0.1 pH units will affect the elution position of deoxyguanosine so that it will interfere with either the deoxycytosine or deoxyadenosine separations; similarly for guanosine in the ribo-series. Longer columns improve the distance of separation between individual components but also increase the time required for the separation. Oligonucleotides and inorganic phosphate co-chromatograph with the mononucleotide fractions.

Fig. 1c shows an identical chromatogram to that in Fig. 1b except for the addition of deoxythymidine to the mixture of ribomononucleotides and mononucleosides



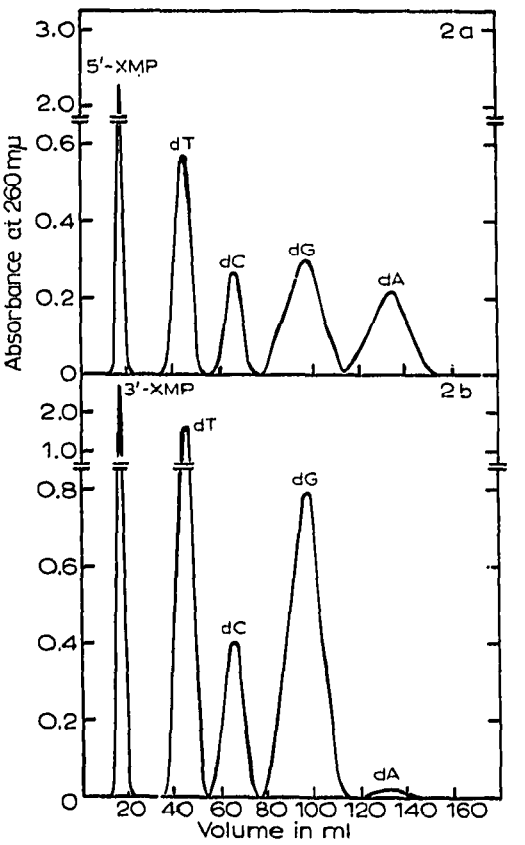


Fig. 1. (a) Chromatography of 5′-deoxynucleotides and deoxynucleosides. A mixture containing the four 5′-deoxynucleotides and four deoxynucleosides was loaded on a 42 × 1.1 cm column and eluted with a 0.3 *M* ammonium formate buffer, pH 9.2.———, 46.80 absorbance units (260 mμ) of mixture in 1.0 ml sample volume. ·····, 2.34 absorbance units (260 mμ) of mixture in 0.05 ml sample volume. (b) Chromatography of 5′-ribonucleotides and ribonucleosides. A mixture containing the four 5′-ribonucleotides and four ribonucleosides was loaded on a 60 × 1.1 cm column and eluted with 0.3 *M* ammonium formate buffer, pH 8.9.———, 36.0 absorbance units (260 mμ) of mixture in 0.6 ml sample volume.·····, 3.0 absorbance units (260 mμ) of mixture in 0.05 ml sample volume. (c) Fractionation of deoxythymidine in the ribonucleoside chromatographic system. To a mixture of 36.0 absorbance units (260 mμ) of 5′-ribonucleotides and ribonucleosides 7.0 A_{260} units of deoxythymidine were added. The total mixture was chromatographed as described in (b). Sample volume: 0.8 ml.

Fig. 2. (a) Chromatography of a snake venom phosphodiesterase digest of the trinucleotides from a DNase 1 digest of *E. coli* K 12 DNA. The fractionation was performed as described in Fig. 1a. Sample volume: 1.0 ml. (b) Chromatography of a spleen phosphodiesterase digest of the trinucleotides from a DNase 1 digest of *E. coli* K 12 DNA. The fractionation was performed as described in Fig. 1a. Sample volume: 2.2 ml.

applied to the column. The deoxythymidine was eluted in a position intermediate between uridine and cytidine and this has an advantage in the sequential analysis of ribo-oligonucleotides which may have small DNA oligonucleotide contaminations in that the deoxythymidine will show up as an individual peak.

In studies of the composition of oligodeoxynucleotides or oligoribonucleotides

TABLE I

QUANTITATIVE ANALYSIS OF THE 5'-NUCLEOTIDES AND NUCLEOSIDES SEPARATED BY CHROMATOGRAPHY ON DOWEX 50

The analyses correspond to the chromatographic separations of the 46.8 A_{260} unit sample shown in Fig. 1a and the 36.0 A_{260} unit sample shown in Fig. 1b. The fractions containing each component were pooled and the total absorbance measured at the pH of the buffer. The figures in parentheses indicate the number of determinations.

Components	*% recovery ± S.E.M.*	*Volume of fraction*
5'-Deoxynucleotides	99.4 ± 0.06 (4)	8.8
dT	99.0 ± 0.03 (4)	15.8
dC	100.0 ± 0.02 (4)	21.5
dG	101.6 ± 0.03 (4)	37.0
dA	98.4 ± 0.07 (4)	43.8
5'-Ribonucleotides	99.5 ± 0.06 (3)	7.4
U	101.0 ± 0.09 (4)	12.4
C	100.8 ± 0.06 (4)	20.0
G	100.0 ± 0.03 (4)	27.8
A	98.9 ± 0.11 (4)	37.0

the first deoxynucleotide or ribonucleotide peaks eluted can be conveniently fractionated into their four components by the procedure of BLATTNER AND ERICKSON[5]. This allows a complete nucleotide/nucleoside analysis within a period of 2 to 2.5 h. The chromatogram shown in Fig. 2a shows results of a phosphomonoesterase and snake venom phosphodiesterase digest of the trinucleotides isolated from a DNase 1 digest. Fig. 2b shows a similar separation of a phosphomonoesterase and spleen phosphodiesterase digest of the same material. Comparing the separations with those obtained in Fig. 1a they are seen to be identical and the salts and enzymes present in the enzyme incubation medium do not interfere with the chromatographic elution. In these experiments the mononucleotides were desalted by sublimation *in vacuo* and subsequently fractionated according to BLATTNER AND ERICKSON[5]. The results are presented in Table II.

Fractionation of all four deoxynucleosides and 5'-deoxynucleotides

A second method of approach for a complete nucleotide/nucleoside separation is shown in Fig. 3. The first elution buffer elutes the four deoxynucleotides and deoxythymidine. Following the appearance of adenylic acid the buffer is changed and a peak of absorbance appears immediately following the application of the second buffer due to the change in salt concentration. A similar effect has been noted previously by KHYM AND UZIEL[11]. The diesterase enzymes do not interfere with the separation. Recoveries from this column are better than 98%.

DISCUSSION

The separation of nucleosides at high pH values on a cation exchanger where the total charge of the nucleosides is nil (for A and C) or partially negative (for G, T and U) is due to preferential non-ionic interactions with the resin[12]. The partial negative charge of the hydroxyl groups of guanosine, deoxyguanosine, uridine and thymidine

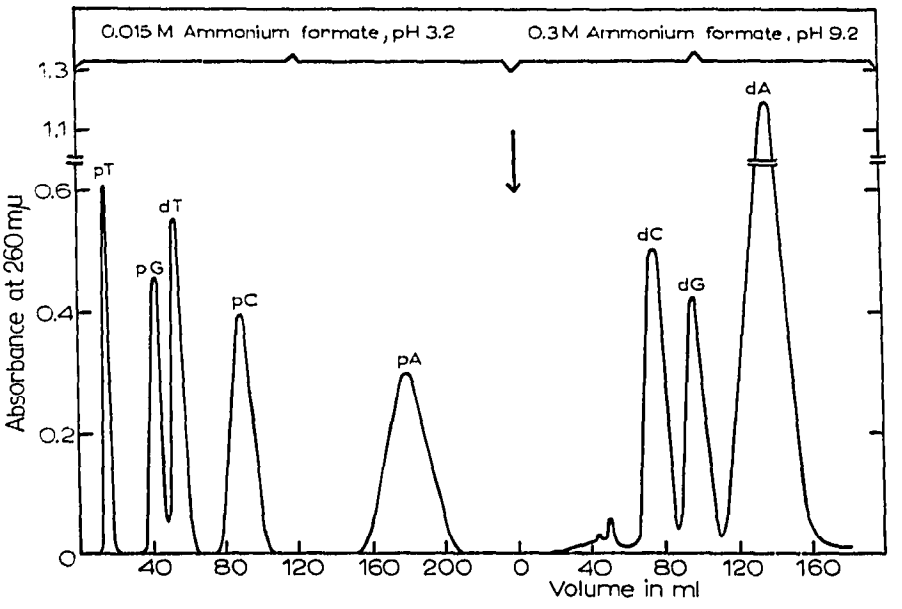


Fig. 3. Separation of all four deoxynucleosides and 5′-deoxynucleotides. A mixture of the eight components totalling 80 absorbance units (260 mμ) was loaded on a 42 × 1.1 cm column in 0.015 *M* ammonium formate buffer, pH 3.2. Sample volume: 1.0 ml. This buffer was then used for elution of the first five components and the second elution buffer, 0.3 *M* ammonium formate, pH 9.2, applied.

TABLE II

ANALYSIS OF THE 5′ AND 3′ ENDS OF THE TRINUCLEOTIDES ISOLATED FROM A DNase I DIGEST OF *E. coli* K 12 DNA

The analysis corresponds to the chromatographic separations shown in Figs. 2a and b. The separated components were pooled and the total absorbance measured at pH 2 (pH 1 for guanine) and the concentrations calculated from the extinction coefficients of the compounds. The 5′-deoxynucleotides from the snake venom phosphodiesterase digest (Fig. 2a) were desalted by evaporation and sublimation *in vacuo* and analyzed according to BLATTNER AND ERICKSON[5].

Components	*5′ end (Fig. 2a)*		*3′ end (Fig. 2b)*	
	μmoles	%	*μmoles*	%
dT	0.275	25.1	0.920	38.0
dC	0.087	7.9	0.410	16.9
dG	0.426	38.9	1.050	43.4
dA	0.307	28.0	0.040	1.7
Total	1.095	99.9	2.240	100.0
pT	0.627	28.3		
pC	0.416	18.8		
pG	0.797	35.9		
pA	0.378	17.0		
Total	2.218	100.0		

at the pH used results in the differential elution of all the components of the mixture. Together with the differences in pK values the additional methyl group of deoxythymidine may explain the increased retention of dT at pH 8.9, a factor used for the separation of this nucleoside from uridine.

The first two systems described, the separation of 3'- or 5'-deoxynucleotides and deoxynucleosides and the separation of 3'- or 5'-ribonucleotides and ribonucleosides, are on column systems which do not require any regeneration. This allows a very fast turn-over of experiments. A complete separation of all four deoxynucleosides and deoxynucleotides on the single column by two buffers at two different pH values requires regeneration of the column and equilibration with the first buffer before re-use. An advantage with all methods is that the enzymes and ions in the digestion mixtures do not interfere with the separations and quantitative analyses. Attempts to determine the actual elution position of the enzyme by using ten-fold excess of enzyme and measuring enzyme activity and protein were unsuccessful. However, the columns have been re-used up to twelve times without repacking. The separation of deoxythymidine from the ribonucleosides provides an easy method for identification of DNA contamination in RNA preparations. The pH at which the elutions occur is in the most stable range for the purine glycosidic links. This is compared to the disadvantage of elution of purine nucleosides and nucleotides with acidic solutions[8]. However, the main advantages of the procedures described above are the rapid separations achieved in 1.5 to 2 h, the quantitative recoveries of the material and the accuracy of the method which allows very small quantities of material to be quantitatively measured (see Fig. 1). The method is currently being applied to the analysis of deoxyribonucleotides from DNase 1 digests (Table II).

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