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A SIMPLE ION-EXCHANGE CHROMATOGRAPHIC PROCEDURE FOR THE ANALYSIS OF THE MAJOR RIBO- AND DEOXYRIBONUCLEOTIDES

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SUMMARY

A method has been described for the separation and estimation of the four major nucleotides of RNA (AMP, GMP, CMP, and UMP) and the four major nucleotides of DNA (dAMP, dGMP, dCMP, and TMP) by Dowex-1 anion-exchange chromatography. The alkaline hydrolysates of RNA and the enzyme hydrolysates of DNA can be used directly without any pretreatment. Each analysis is completed within 60–90 min with the minimum of manipulation steps. Complete recovery is obtained with a wide range of nucleotide concentrations (50–500 mμmoles of each nucleotide).

INTRODUCTION

Analysis of base composition is an essential first step in the characterization and sequencing of nucleic acids. For this purpose, the nucleic acids are generally hydrolyzed into monomeric units by enzymatic or chemical means and the monomers are then separated and estimated using one of many available techniques based on paper or column chromatography. Chromatography and electrophoresis on paper or similar supports can be used to separate and identify micro-amounts of nucleic acid hydrolysates, but subsequent recovery of the separated nucleotides for quantitation would require time-consuming elution procedures. Column chromatography avoids some of these problems and ion-exchange resins have become particularly popular after the initial successful use of these materials by COHN AND VOLKIN[1]. These authors used buffers containing mixtures of formic acid and ammonium formate to separate ribonucleotides on Dowex-1 formate columns. In spite of the obvious advantages of using such a volatile eluant in the recovery of nucleotides from the eluting medium, the method suffers from the necessity of using large volumes of eluant and a long elution time.

In recent years, several modifications have been introduced in the original ion-exchange chromatography of nucleotides, using a variety of anion- and cation-exchange resins, step-wise or continuous gradient elutions, miniaturized columns,

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highly sensitive UV recording spectrophotometers and high-pressure chromatography[2-9]. This work has resulted in the development of ingenious methods, some of which fulfil certain special analytical needs while others suffer from procedural complications or require equipment not readily available in many laboratories. In the present communication, we present a chromatographic technique for the separation and determination of the eight common nucleotides of DNA and RNA, rapidly and with the minimum of manipulation.

MATERIALS AND METHODS

Materials

Dowex-1 X-8 (200–400 mesh, spherical granules) was obtained from J. T. Baker Chemical Company, Phillipsburg, N.J., U.S.A. The 2'(3')-ribonucleotides and 5'-deoxyribonucleotides were purchased from P-L Biochemicals, Milwaukee, Wisc., U.S.A. DNase 1 (pancreatic crystalline) and snake venom phosphodiesterase were from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Highly polymerized yeast RNA was from Calbiochem, Los Angeles, Calif., U.S.A. DNA from rat brain was prepared as described previously[10].

Pretreatment of the resin

The chloride form of Dowex-1 X-8 was treated as follows, prior to packing of the column. The resin was washed three times with 10 volumes of water followed by two washings with 10 volumes of 2 *M* NaOH. Each time, the resin was allowed to settle for 30 min in the liquid and the unsettled particles were discarded by decantation. The resin was then taken up in a buchner funnel and washed several times with water until the pH of the filtrate was neutral. The resin was transferred to a beaker and washed twice with 10 volumes of 2 *M* HCl, with decantation each time to remove the finer particles as described above. The excess of acid was removed by washing the resin on a buchner funnel with water and the resin was then converted to the formate form by washing with 1 *M* sodium formate. The formate treatment was continued until the washings gave no precipitate with acidified silver nitrate solution. The excess of sodium formate in the resin was removed by thorough washing with water.

Preparation of the column

The column consisted of a Pyrex glass tube of 90 mm height and 6 mm I.D. The resin was mixed with water to make a thin slurry which was poured into the column in small batches by means of a pasteur pipette, taking care to avoid trapping of air bubbles. The height of the resin bed was 6 cm, with a bed volume of approx. 1.7 ml. The resin was supported on a flat, thin piece of glass wool placed at the outlet of the column. The top of the column was connected to a Buchler peristaltic pump which was, in turn, connected to a multichamber gradient former (Technicon Autograd). The first three chambers nearest to the outlet of the autograd were used for the eluting gradient.

Adsorption and elution of nucleotides

Synthetic nucleotides as well as nucleotides derived from DNA and RNA were

used in these experiments. DNA (rat brain) was hydrolyzed to 5'-deoxyribonucleotides by successive incubations with pancreatic deoxyribonuclease I and snake venom phosphodiesterase according to McCALLUM AND WALKER[11]. RNA (yeast, highly polymerized) was hydrolyzed to a mixture of 2'- and 3'-ribonucleotides by incubating with 0.3 *M* KOH at 37° for 18 h[12].

5'-Derivatives of the deoxyribonucleotides, dAMP, dGMP, dCMP, and TMP were dissolved in water and the pH of the solutions was adjusted to 7.0. The ribonucleotides, AMP, GMP, CMP, and UMP (all mixtures of 2'- and 3'-phosphates) were dissolved in 0.3 *M* KOH. The solutions were each made up to 0.5 m*M*. Appropriate volumes of the synthetic nucleotides or hydrolysates of nucleic acids were mixed with 30 ml of 0.1 *M* ammonium formate. The mixture was passed through the Dowex-1 column and the column was washed with 20–25 ml of distilled water.

The deoxyribonucleotides were fractionated as follows. Formic acid (0.15 *M*) was passed through the column until dCMP was completely eluted. This required approx. 10–12 ml of the formic acid. Then the column was connected to the gradient former containing 40 ml of 0.15 *M* formic acid in the first chamber nearest to the outlet, 40 ml of 4 *M* formic acid in the second chamber and 40 ml of 2 *M* formic acid in the third chamber. The interconnecting passages between the chambers were opened and the contents of each chamber mixed continuously by light mechanical agitation. Elution was carried out at the rate of 1 ml/min and the eluate was monitored continuously at 257 mμ by means of an LKB Uvicord. 1-ml fractions of the eluate were collected in separate tubes.

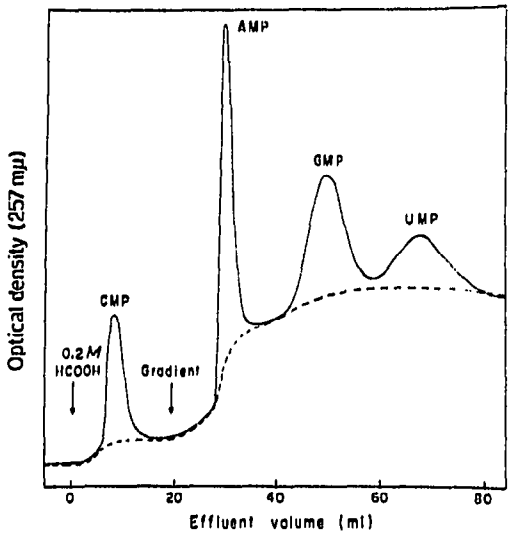
The ribonucleotides were fractionated as follows. CMP was eluted first by passing approx. 20–25 ml of 0.2 *M* formic acid. The column was then connected to the gradient former containing 40 ml of 0.2 *M* formic acid, 40 ml of 4.5 *M* formic acid and 40 ml of 2.5 *M* formic acid, respectively in the first three chambers nearest to the outlet. The rest of the protocol was identical to that for the ribonucleotides.

Determination of recovery of the nucleotides

The fractions corresponding to each peak were pooled together. The optical density at 260 mμ was determined either directly on the pooled fractions or after lyophilizing the eluate corresponding to each nucleotide and redissolving the residue in water or 1 *M* HCl. Since formic acid itself has considerable optical density in the UV region, the gradient was passed through the column without any adsorbed nucleotide material and the fractions of formic acid corresponding in position to the nucleotide peaks, were collected to serve as blanks. The $O.D._{260}$ units of each nucleotide recovered from the column in the above manner were compared with the $O.D._{260}$ units originally placed on the column.

Other analyses

The individual nucleotides, before and after passage through the column, were scanned for UV absorption spectrum using an Unicam spectrophotometer. Any possible degradation or mutual contamination of the eluted nucleotides was examined by paper electrophoresis using a Savant high-voltage electrophoresis apparatus, using ammonium formate–formic acid buffer as the solvent system.



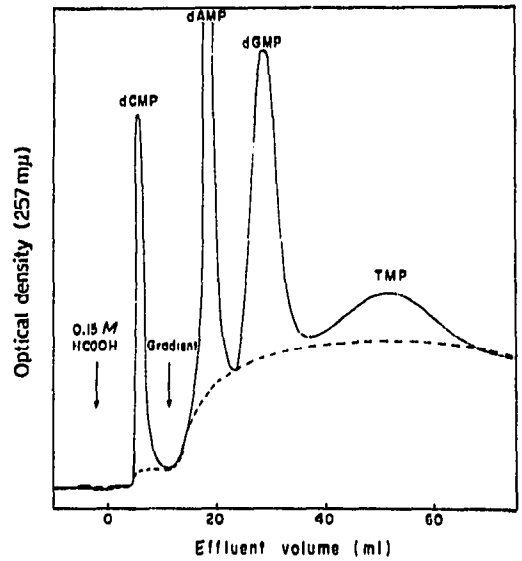


Fig. 1. Separation of a mixture of 2′- and 3′-phosphates of adenosine, guanosine, cytidine, and uridine by Dowex-1 anion-exchange chromatography. The amount of each nucleotide placed on the column was equal to 0.25 μmoles. Immediately after all nucleotides were eluted, the column was regenerated by passing 4 *M* formic acid followed by water until the eluate was neutral. A gradient of formic acid identical to the first was passed through the column a second time in order to obtain the changes in optical density due to the gradient alone. The continuous line represents the optical density changes in the presence of added nucleotides and the broken line represents the optical density changes in the absence of added nucleotides.

Fig. 2. Separation of a mixture of 5′-phosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine by Dowex-1 anion-exchange chromatography. The experimental protocol was the same as in Fig. 1.

RESULTS

Figs. 1 and 2 show the separation of the four common ribonucleotides (2′,3′-phosphates of adenosine, guanosine, cytidine, and uridine) and the four common deoxyribonucleotides (5′-dAMP, 5′-dGMP, 5′-dCMP, and 5′-TMP) on Dowex-1 anion-exchange columns. The absorption peaks were well separated and compact, except for a slight broadening of the UMP and TMP peaks. Both 2′- and 3′-nucleotides of each base eluted together. The above separation was made using mixtures of pure nucleotides. Similar results were obtained using an alkaline hydrolysate of RNA(KOH) and an enzymatic digest of DNA (DNase 1 and snake venom phosphodiesterase).

The base lines of the CMP and dCMP peaks were close to zero optical density, but the base lines of other peaks remained at a high level. This was due to the fact that formic acid used in the elution gradient had a high UV absorption. The contribution of formic acid to the optical density of each peak was measured by passing the gradient through the column under conditions identical to the normal procedure except that the nucleotide mixture was omitted from the column. The profiles of the absorption due to formic acid in the two gradients are also shown in Figs. 1 and 2.

Because of the varying optical density of the formic acid in the gradient, the recovery of the nucleotide from the column was estimated either by removing the

formic acid from the eluates by lyophilization or by reading the $O.D._{260}$ of the eluates against the corresponding formic acid blanks. When the lyophilized fractions were redissolved in water or 1 *M* HCl and the optical density of this solution read at 260 mμ, normal recovery values were obtained for the first two peaks of each nucleotide mixture (CMP, AMP, dCMP and dAMP), but the recoveries of GMP, UMP, dGMP and TMP were 25–40% higher than the input. We have not investigated the reasons for the abnormal recoveries obtained with the ribo- and deoxyribonucleotides of guanine and uracil (thymine).

Lyophilization removes the formate ion only when it is in the form of acid or ammonium salt, which are both volatile; non-volatile formate, if present, is not removed and hence could be expected to contribute to the optical density. It is also possible that, at the high acid concentrations at which these nucleotides are eluted, some non-volatile, UV absorbing material is introduced. However, paper electrophoresis of any of the ribo- and deoxyribonucleotides recovered from the column failed to indicate the presence of any UV absorbing spots other than those due to the nucleotides themselves. UV spectra of the original acid-eluted nucleotides were also found to be identical.

Normal recoveries of all nucleotides were found when the $O.D._{260}$ of the eluates from the column were taken directly without lyophilization, using corresponding formic acid blanks obtained from the column. Equally good results were obtained by using the following simplified procedure. The fractions corresponding to each peak were pooled together and the solution was titrated against NaOH of known

TABLE I

RECOVERY OF ADDED RIBONUCLEOTIDES AFTER DOWEX-1 ANION-EXCHANGE CHROMATOGRAPHY

Nucleotide	*Added to column (O.D. at 260mμ)*[a]	*Recovered from column (O.D. at 260mμ)*[a]
AMP	1.64 ± 0.06	1.61 ± 0.01
GMP	3.51 ± 0.17	3.49 ± 0.15
CMP	2.76 ± 0.09	2.88 ± 0.12
UMP	2.23 ± 0.12	2.22 ± 0.10

[a] The values represent the mean of three separate determinations. The optical densities of the column eluates were corrected for the contribution of formic acid as described in RESULTS.

normality in order to determine the concentration of formic acid present in the solution. The $O.D._{260}$ of this solution was taken against sodium formate solution of identical concentration. The recovery was calculated from this optical density and the total volume of the solution.

Table I shows the recoveries of the four ribonucleotides obtained by the above procedure. The recoveries were in all cases around 100%. Similar results were obtained using a mixture of deoxyribonucleotides.

DISCUSSION

Dowex-1 anion-exchange resin has been used by several workers for the separation of nucleotides, nucleosides, and oligonucleotides derived from nucleic acids[1–4,13].

The modifications of the original method of COHN AND VOLKIN[1] have been aimed among others at reducing the time of analysis, simplifying the various steps in the procedure, separating the minor nucleotides present in nucleic acids, scaling down the amount of nucleotide material to micro- and ultramicro-levels, etc. This has resulted in the development of several sophisticated techniques and apparatus. Our objective was to find a simple and rapid procedure for the routine determination of the base composition of DNA and RNA for a wide range of availability of these nucleic acids. The method of KATZ AND COMB[2] permits such a determination, but it is applicable only to RNA nucleotides and it involves such operations as step-wise elution and use of two different columns. LUKASOVA *et al.*[3] avoid this problem by using a single column and elution with a gradient of chloride ions. However, this method results in a separation of the 2',3'-isomeric phosphates of adenosine and guanosine. HURLBERT AND FURLONG[13] have described procedures for the separation of 5'-ribonucleotides and 5'-deoxyribonucleotides on Dowex-1 formate columns, but again the method involves the step-wise elution of deoxyribonucleotides and use of large volumes of eluants.

The procedure that we have presented in this communication has the following advantages: (a) the same column can be used for the separation of either the ribonucleotides or deoxyribonucleotides using two different elution systems; (b) both the elution systems are composed of formic acid gradients and so the nucleotides can be recovered from the eluates, when necessary, by lyophilization; (c) nucleic acid hydrolysates prepared according to the most common procedures, *viz.* by treatment of DNA with DNase and snake venom phosphodiesterase and by treatment of RNA with 0.3 *M* KOH can be directly used on the column without any pretreatments; (d) the 2'- and 3'-nucleotide isomers produced by alkaline hydrolysis of RNA are not separated on the column, so that each purine and pyrimidine base is represented by a single peak; (e) the amounts of nucleotides that can be separated on the column can be varied over a wide range (50–500 mμmoles of each nucleotide) with complete quantitative recovery; (f) the column is small and simple to construct and each analysis can be completed within 60–90 min, at room temperature and atmospheric pressure. It should be stressed, however, that the presence of considerable proportions of methylated and other minor nucleotides, such as in the case of transfer RNAs, would give variable higher values for the major nucleotides since they would be distributed in the four peaks of the chromatogram.

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