BBA 96110

CHROMATOGRAPHY OF NUCLEIC ACIDS ON HYDROXYAPATITE
III. CHROMATOGRAPHY OF RNA AND POLYRIBONUCLEOTIDES

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(Received September 9th, 1968)

SUMMARY

The chromatographic behaviour on hydroxyapatite columns of ribosomal and viral RNA, of transfer RNA and of several biosynthetic polyribonucleotides has been investigated in view of obtaining information useful for our understanding of the relationship between the secondary structure of polynucleotides and their elution molarity.

Single-stranded, random-coiled polynucleotides are eluted at lower phosphate molarities than double-stranded, rigid polynucleotides. All double-stranded polynucleotides are eluted at about the same phosphate molarity, 0.20–0.22 M. Triple-stranded 2 poly U–poly A is eluted at a higher molarity (about 0.45 M).

The chromatographic behaviour of bases, nucleosides, nucleoside mono- and polyphosphates, and oligonucleotides has also been studied, mainly for elucidating the mechanism of adsorption of polynucleotides on hydroxyapatite.

INTRODUCTION

The chromatographic behaviour on hydroxyapatite columns of native and denatured DNA has been described in the preceding articles[1,2] and elsewhere[3]. The results obtained clearly indicate that the elution molarity of DNA is strongly dependent upon its secondary structure, and therefore further investigations were conducted on the relationship between the chromatographic behaviour of polynucleotides on hydroxyapatite columns and their secondary structure, using several different RNA's as well as synthetic polyribonucleotides. These studies have been much less extensive than those just reported on DNA, their purpose being limited to obtaining further information relevant to our understanding of the physicochemical basis of the polynucleotide–hydroxyapatite interaction. The results reported here are also useful, however, in connection with separation problems encountered in the area of polyribonucleotides. Since this paper will also deal with the mechanism of adsorption of polynucleotides on hydroxyapatite columns, results obtained with nucleic acid derivatives will be mentioned here.

MATERIALS AND METHODS

These were described in the first paper of this series[1].

RESULTS

Chromatography of high-molecular weight RNA's

Using the stepwise elution technique, ribosomal RNA from yeast and from Ehrlich ascites tumour cells and RNA from tobacco mosaic virus, turnip yellow mosaic virus, and alfalfa mosaic virus were all eluted in two peaks at 0.15 M and 0.20 M potassium phosphate buffer (Fig. 1A). The relative proportions of the two fractions seem to depend solely upon the charge/column bed ratio, higher ratios yielding larger 0.15 M fractions. This result, which recalls the findings obtained using the stepwise elution technique on native DNA (see the first paper of this series), suggests that the 2nd peak a "false" peak. In agreement with this conclusion, when elution was carried out with a molarity gradient high-molecular weight RNA's were all eluted as single peaks (Fig. 1B), at a molarity of 0.15 M potassium phosphate buffer.

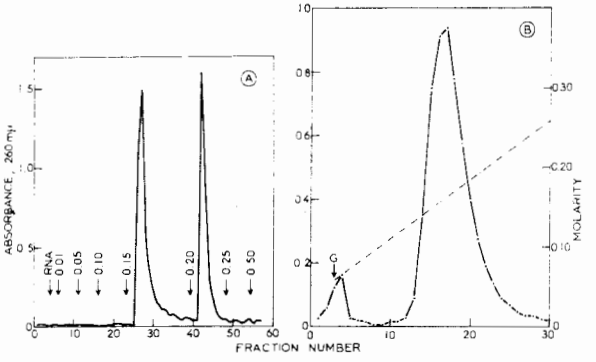


Fig. 1. A. Chromatography of ribosomal RNA from Ehrlich ascites tumour on a 1.3 cm × 6 cm hydroxyapatite column. 8 ml of an RNA solution having an $A_{260\ m\mu} = 3.12$ were loaded on the column at fraction number four. 3.2 ml-fractions were collected, using the stepwise elution technique. Recovery was 100 %. B. Chromatography of 20.8 $A_{260\ m\mu}$ units of ribosomal RNA from yeast. In this and all following figures, loading took place at fraction number zero. 1 cm × 10 cm hydroxyapatite columns were used in the experiments shown, except where otherwise stated. A molarity gradient of potassium phosphate buffer was started at the fraction marked with G. 2.9-ml fractions were collected. Recovery was 91 %. This experiment was performed at 4°.

No detailed investigation has been done, so far, in our laboratory, on the dependence of the elution molarity of high-molecular weight RNA's upon temperature or molecular weight, yet no supporting evidence was obtained in our work for the claim[4,5] that hydroxyapatite columns fractionate high-molecular weight RNA on the basis of its molecular weight.

It should be pointed out that, in several cases, the sedimentation coefficients of high-molecular weight RNA's eluted from hydroxyapatite columns were found to be lower than those of the starting materials (see, for instance, ref. 6). This decrease in sedimentation coefficient might be due to the fact that hydroxyapatite columns separate from each other the large polynucleotide fragments which form RNA molecules containing "hidden breaks". That the columns do not cause by themselves any breakage of large RNA molecules is shown by the good recovery of infectivity obtained when tobacco mosaic virus-RNA is chromatographed on hydroxyapatite (L. PINCK AND L. HIRTH, personal communication).

Chromatography of double-stranded RNA

The replicative forms and the replicative intermediates[7] of viral plant RNA's are eluted from hydroxyapatite columns at the same molarity as native DNA[8], and can therefore be readily separated from plant ribosomal RNA. This separation[8] is analogous to the separation of denatured and native DNA[2,9].

Chromatography of transfer RNA

Transfer RNA from *Escherichia coli* or yeast is eluted by a potassium phosphate buffer molarity gradient at about 0.13 M (ref. 9). The resolution of different tRNA species was not attempted in our laboratory. Investigations on this particular problem have been published by other authors[10–13] who have shown the great usefulness of hydroxyapatite columns for this type of fractionation.

The fractionation of different tRNA's is not unexpected on the basis of what is known about the discriminating power of hydroxyapatite with respect to different secondary and tertiary structures of polynucleotides (ref. 9, and DISCUSSION).

Chromatography of polyribonucleotides

Polyuridylic acid is eluted at a molarity of 0.10 M potassium phosphate buffer at 25° (Fig. 2A) under conditions where it is completely devoid of any secondary structure[14], but at 0.15 M potassium phosphate buffer at 4° (Fig. 2B), where the polynucleotide has some sort of helical secondary structure, as shown by its hypochromism and increase in positive optical rotation[14,15]. The recovery of poly U from the columns has always been found to be complete. In contrast to poly U, poly A is eluted, at room temperature, in a broad peak centered at 0.22–0.25 M potassium phosphate buffer (Fig. 3A). The recovery of poly A has never been found to be complete when the usual linear molarity gradients 0.001–0.5 M potassium phosphate buffer were used. The usual recoveries were between 60 and 85 %. It may be recalled here that at room temperature, polyadenylic acid is known to have a structure which

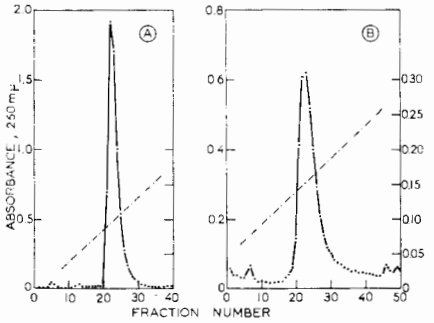


Fig. 2. Chromatography of polyuridylic acid. 2.9-ml fractions were collected. (A) Experiment performed at 25°; 20 $A_{260\ m\mu}$ units were loaded; recovery was 98 %. (B) Experiment performed at 4°; 6.5 $A_{260\ m\mu}$ units were loaded, recovery was 100 %. See also legend of Fig. 1.

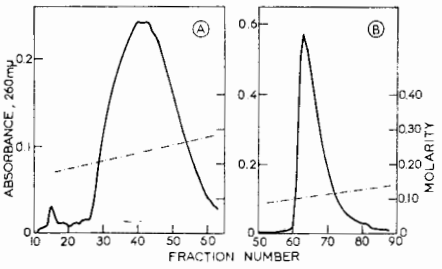


Fig. 3. Chromatography of: (A) 20.9 $A_{260\ m\mu}$ units of polyadenylic acid; 3-ml fractions were collected. Recovery was 81 %. (B) 9.9 $A_{260\ m\mu}$ units of polycytidilic acid. 2.9-ml fractions were collected. Recovery was 100 %. See also legend of Fig. 1.

is random with respect to total conformation, but ordered in terms of short-range interactions, the ordered regions having a single-stranded, stacked, helical structure[17–19]. Polycytidylic acid is eluted, at room temperature, at a molarity of about 0.12 M phosphate (Fig. 3B). At 4°, poly C was eluted at higher phosphate molarities than at 25°. The recovery of poly C from the columns was complete. Poly I gave very irregular results when chromatographed on hydroxyapatite columns; its behaviour was characterized by extremely low recoveries.

Summing up these results, the two polypyrimidinic acids showed good recoveries from the columns; they were eluted in narrow peaks and their elution molarity was strongly influenced by temperature. Of the two polypurinic acids, poly A was eluted in a broad peak with incomplete recovery and poly I was eluted with very low recoveries. Clearly, further investigations need to be made on the chromatographic behaviour of synthetic polyribonucleotides, and particularly of poly A and poly I, using different experimental conditions. A first step in this direction is shown in Fig. 4; the 4 polynucleotides just mentioned were dissolved in 0.01 M potassium phosphate buffer containing 1 % formaldehyde and heated up to 100° for 5 min.

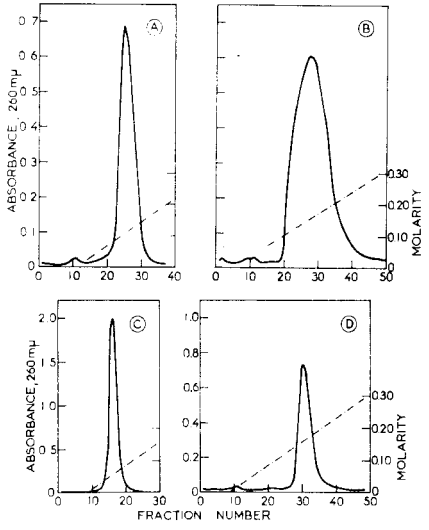
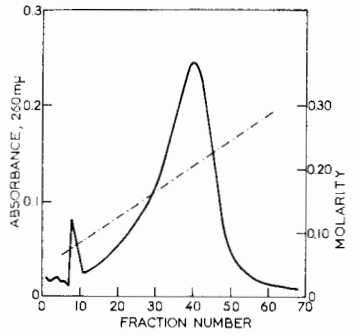


Fig. 4. Chromatography of: (A) 11 $A_{260\ m\mu}$ units of polycytidilic acid. (B) 23.3 $A_{260\ m\mu}$ units of polyadenylic acid. (C) 19.5 $A_{260\ m\mu}$ units of polyurydilic acid. (D) 11.7 units of polyinosinic acid. Polyribonucleotides were heated up to 100° for 5 min and fast cooled in 0.01 M potassium phosphate buffer containing 1 % neutralized formaldehyde; this was also present in the eluting buffers. 1 cm × 20 cm hydroxyapatite columns were used. 3-ml fractions were collected. Recoveries were 100 %.

After a rapid cooling, the polynucleotides were chromatographed at room temperature on hydroxyapatite columns using potassium phosphate buffer containing 1 % formaldehyde. Recoveries were complete in all cases and the elution molarities were 0.05 M potassium phosphate buffer for poly U, 0.12 M for poly C, 0.16 M for poly I and 0.17 for poly A. These results are presented here to show how hydroxyapatite columns discriminate among different single-stranded structures, and to help our

understanding of the fractionation of single-stranded, heat-denatured and formaldehyde-reacted DNA according to its base composition.

The artificial complexes of poly U and poly A were also examined under conditions where double-stranded poly A–poly U, or triple-stranded 2 poly U–poly A were formed. The double-stranded complex was eluted at a molarity only slightly higher than poly A, whereas the triple-stranded complex was eluted at a much higher molarity, in the 0.45–0.50 M potassium phosphate buffer region[9]. The double-stranded poly I–poly C complex was eluted at a molarity close to 0.20 M, like poly A–poly U (Fig. 5).



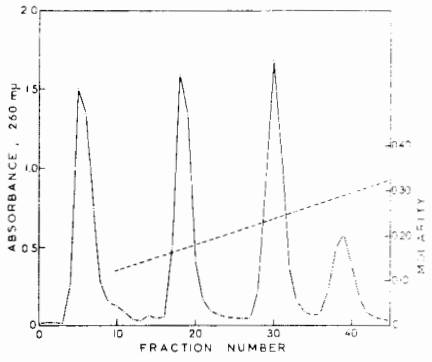


Fig. 5. Chromatography of 14.0 $A_{260\,m\mu}$ units of polyinosinic acid + polycytidylic acid. 2.9-ml fractions were collected. Recovery was 100 %. See also legend of Fig. 1.

Fig. 6. Chromatography of a mixture of AMP, ADP, ATP, and adenosine tetraphosphate. Recoveries were 133, 113 and 50 %, respectively, owing to the fact that ADP, ATP, and, to a much larger extent, adenosine tetraphosphate, were contaminated by the lower phosphates, as indicated by separate experiments. The overall recovery was 101 %. 3-ml fractions were collected. Elution was carried out with a potassium phosphate buffer molarity gradient, 0.001 M–0.5 M (100 + 100 ml). See also legend of Fig. 1.

Chromatography of oligonucleotides

The bulk of the oligonucleotides of RNA "core", the water-undialyzable oligonucleotides obtained by digesting RNA with pancreatic ribonuclease, was eluted at phosphate molarities lower than 0.02 M (ref. 9), in agreement with the early report[2]-, confirmed in our laboratory, that oligonucleotides obtained from DNA by pancreatic deoxyribonuclease digestion are eluted from hydroxyapatite columns at very low phosphate molarities. A small fraction of RNA "Core" was eluted at 0.10 M potassium phosphate buffer. This might by formed by larger fragments endowed with a more ordered structure (see DISCUSSION). The fractionation of large oligonucleotides on hydroxyapatite columns, both in the presence and in the absence of urea or formaldehyde, appears to be very promising, as indicated by results obtained by MUNDRY[21] and ourselves (work to be published).

Chromatography of bases, nucleosides and nucleoside mono- and polyphosphates

Some results, already briefly presented elsewhere[22], on the chromatography of nucleic acid derivatives on hydroxyapatite will be recalled here because of their significance for the interpretation of the chromatographic behaviour of nucleic acids.

Purine and pyrimidine bases, ribo- and deoxyribonucleosides and coenzyme derivatives, like thiamine and riboflavin, are not retained by the columns equilibrated with 0.001 M potassium phosphate buffer. Nucleoside monophosphates, thiamine monophosphate, and riboflavin 5'-phosphate are also eluted by 0.001 M buffer, but are slightly retarded. Nucleoside polyphosphates on the other hand, are strongly adsorbed and need increasingly high molarities of phosphate buffer to be eluted (Fig. 6).

Similar experiments showed that other nucleoside 5'-diphosphates and thiamine pyrophosphate were eluted at the same phosphate molarity as ADP, whereas nucleoside 5'-triphosphates were eluted like ATP. The only exception found so far is that GDP and GTP and, to a smaller extent, IDP and ITP, are eluted at slightly higher phosphate molarities than the other di- and triphosphates, respectively.

Compounds in which the pyrophosphate has no free secondary group, like ADP-ribose, NAD, FAD, UDP-glucose, are not retained by the column equilibrated with 0.001 M potassium phosphate buffer; NADP and coenzyme A are retarded only slightly more than nucleoside monophosphates.

DISCUSSION

The results presented in this paper relate mainly to two problems: the mechanism of adsorption and elution of polynucleotides on hydroxyapatite columns, and the relationship between the elution molarities of polynucleotides and their secondary structure. The present discussion will be limited to a qualitative treatment of these two problems.

Concerning the first point, the following results indicate that adsorption of polynucleotides on hydroxyapatite takes place essentially because of the interaction between the negative phosphate groups of the polynucleotides and the positive calcium ions on the surface of hydroxyapatite crystals, with no direct intervention of the bases and the sugars.

(a) Electrophoresis of hydroxyapatite crystals, prepared according to TISELIUS, HJERTEN AND LEVIN[23], was carried out in 0.001 M potassium phosphate buffer using a U-shaped tube, of the type used for determining the electrophoretic mobility of red blood cells. It was shown that the crystals move towards the cathode and have therefore a positive charge. The determination of the isoelectric point was not attempted; it is known from the work of MATTSON *et al.*[24] that hydroxyapatite crystals behave like amphoteric colloids and that the isoelectric point of hydroxyapatite varied, for different preparations examined by those authors, from 6.5 to 10.2. It has also been found in our laboratory that the sedimentation rate of hydroxyapatite crystals suspended in 0.001 M potassium phosphate buffer is greatly increased by the addition of DNA, probably because of a decrease in electrostatic repulsions among the crystals.

(b) Treatment of hydroxyapatite crystals with dilute calcium-complexing agents, like EDTA and citrate, reduces their adsorption capacity for nucleic acids. An identical observation had been reported for proteins by TISELIUS, HJERTEN AND LEVIN[23]. Carboxylic compounds which have a weaker affinity than EDTA and citrate for calcium ions, like acetate ions, may be used as eluents (work to be published).

(c) Our results on the chromatography of nucleoside mono- and polyphosphates and phosphorylated coenzyme derivatives and their organic moieties, clearly show

that the elution molarity of these low-molecular weight compounds is dependent only upon their phosphate groups: for instance, all pyrophosphates and all triphosphates elute at the same phosphate molarity, independent of the nature of the organic molecules to which they are bound. The only exceptions found so far, guanosine di- and triphosphates and, to a lesser extent, inosine di- and triphosphates, which are eluted at higher phosphate molarities, very probably show this behaviour because of intermolecular associations[25], leading to high local concentrations of phosphate groups. The exclusive implication of phosphate groups in the interaction with hydroxyapatite crystals without any interference by the bases or the sugars can be also inferred from the results obtained with polynucleotides (see below).

(d) Phosphoproteins have a much higher affinity for hydroxyapatite than non-phosphorylated proteins[26]. In this latter case, the chemical groups interacting with hydroxyapatite are the negatively-charged carboxyl groups[27] which also have, even if to a lesser degree than phosphate groups, a high affinity for calcium ions.

(e) Elution of polynucleotides from hydroxyapatite is caused by the progressive increase of the molarity of the eluting phosphate buffer and appears to be due to a specific competition between the phosphate ions of the eluting buffer and the phosphate groups of polynucleotides for the calcium ions of hydroxyapatite and not simply to an increase in ionic strength. In fact, it has been shown in the first paper of this series[1] that elution can be performed at a practically constant ionic strength. Native DNA is eluted at the same phosphate molarity independent of the ionic strength of the eluting buffer, provided that the added salt does not repress significantly the ionization of phosphate, in which case the elution molarity increases.

Concerning the relationships between the elution molarities of polynucleotides and their secondary (and tertiary) structures, the results presented in this series of papers lead to the following conclusions.

(a) In the case of polynucleotides endowed with rigid structures, it is evident from our results that all polynucleotides possessing a double-stranded structure, like native, double-stranded DNA, replicative RNA, poly A–poly U, and poly I–poly C, are eluted at about the same molarity, at 0.20–0.22 M potassium phosphate buffer, independent of their base composition. Slight differences in the elution molarities of double-stranded polynucleotides exist and be possibly related to differences in their secondary structures; such seems to be the explanation for the separation of nuclear and mitochondrial DNA's from yeast[28].

The only triple-stranded polynucleotide examined, 2 poly U–poly A, is eluted at a much higher phosphate molarity about 0.45 M potassium phosphate buffer. This higher elution molarity may possibly be explained by the fact that triple-stranded polynucleotides have a higher linear charge density than double-stranded polynucleotides, and therefore a larger number of phosphate groups per unit length is available for interaction with hydroxyapatite, and/or by the fact that the distribution of these groups in 2 poly U–poly A matches the distribution of adsorbing sites on hydroxyapatite better than that of phosphates of double stranded polynucleotides.

It is quite possible that the theory developed in this laboratory by KAWASAKI[28] for the chromatography of rigid macromolecules on hydroxyapatite columns is valid for the case of rigid polynucleotides.

(b) As far as polynucleotides endowed with a random-coil structure are concerned, their chromatographic behaviour needs to be investigated under a much

wider set of experimental conditions than in the present work, since their structure is strongly dependent upon ionic strength and temperature in the range used in chromatography. It is possible that hydroxyapatite columns will become in the future a useful tool for the study of partially or fully ordered, single-stranded polynucleotides.

A very important point which seems, however, to be firmly established is that, in all cases, flexible, randomly-coiled polynucleotides are eluted from hydroxyapatite columns by lower phosphate molarities than rigid, helical polynucleotides. This lower affinity for hydroxyapatite can be seen by comparing the elution molarities of native and denatured DNA, and viral RNA's and their replicative forms. Interestingly enough, ordered poly U also is eluted at a higher molarity than disordered poly U.

The property of hydroxyapatite columns of discriminating ordered, rigid structures from disordered, flexible ones is quite general, since in the case of proteins the disruption of their secondary and tertiary structure also causes a drastic decrease of their affinity for hydroxyapatite[27]. A general explanation for this phenomenon is that phosphate or carboxyl groups, which were available for the interaction with adsorbing sites on hydroxyapatite in the rigid, ordered structures, greatly decrease in number on the "outer surface" of the randomly-coiled, denatured nucleic acids or proteins. A similar explanation may hold for the decrease affinity for hydroxyapatite observed for the twisted circular form compared to both the linear open and the linear circular forms of polyoma virus DNA[30]. Furthermore, local concentrations of phosphates or carboxyls due to the existence of rigid secondary and tertiary structures disappear upon denaturation. An example of the importance of charge distribution in the chromatography on hydroxyapatite columns is given by the strikingly different behaviour of compounds, like nucleoside triphosphates and trinucleoties, which have the same net charge; nucleoside triphosphates are eluted by 0.2 M potassium phosphate buffer, whereas trinucleotides are eluted by 0.001 M potassium phosphate buffer. This phenomenon, which allows separations which cannot be obtained on other types of columns, seems to be due to the different possibilities of matching the phosphate charges with fixed distribution of calcium sites on the hydroxyapatite surface.

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