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CHROMATOGRAPHY OF NUCLEIC ACIDS ON HYDROXYAPATITE

I. CHROMATOGRAPHY OF NATIVE DNA

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SUMMARY

Using the stepwise elution technique, native doublestranded DNA is eluted by 0.20 M and 0.25 M potassium phosphate buffer, pH 6.8: occasionally minor fractions are eluted by 0.30 M and 0.50 M phosphate. Rechromatography experiments showed that the multipeak pattern obtained is an artifact. Indeed, when using the gradient elution technique all native DNA preparations investigated in the present work were eluted as single peaks.

A different chromatographic behaviour was shown by singlestranded DNA from Φ X174 phage, yeast mitochondrial DNA, and glucosylated DNA.

No significant changes in the physical, chemical or biological properties of native DNA are caused by the adsorption-elution process. The native DNA's studied here were not fractionated by hydroxyapatite columns as far as their base composition and biological properties are concerned. A very limited extent of fractionation on the basis of molecular weight was observed. It was shown that elution can be performed at constant ionic strength and in the presence of a variety of organic molecules.

INTRODUCTION

Several different kinds of chromatographic columns have been used in recent years to fractionate nucleic acids: histone–kieselguhr[1,2], methylated serum albumin–kieselguhr[1,3–10], ECTEOLA-cellulose[11,12], Dowex 1 (ref. 13), Amberlite IRC-50 (refs. 14–16), DEAE-cellulose[17,18], benzoylated DEAE-cellulose[19].

Hydroxyapatite columns, originally developed by TISELIUS, HJERTEN AND LEVIN[20] for protein chromatography, have been first used with nucleic acids by SEMENZA[21] in Tiselius laboratory and MAIN *et al.*[22–24].

Work on the chromatography of nucleic acids on hydroxyapatite* columns was started in this laboratory in 1959, because the results previously obtained by BER-

* Hydroxyapatite, not hydroxylapatite, is the name recommended by WYCKOFF[61] since "hydroxyl" implies the derivative being named after the substituted ion, a usage which is not observed in the corresponding fluorine and chlorine derivatives, "fluorapatite" not *e.g.* "fluoridapatite".

NARDI AND COOK[25] in the fractionation of phosphoproteins suggested that hydroxyapatite might be a useful substance in the chromatography of polynucleotides.

Short preliminary reports on the chromatography of native DNA[26,27], ribosomal RNA[28], heat-denatured DNA from calf thymus and chicken erythrocytes[29], nucleoside phosphates and phosphorylated coenzymes[30], heat-denatured transforming DNA from *Haemophilus influenzae*[31], viruses and viral DNA[32], as well as a brief general report[33], have already been published. More recent work from this laboratory has concerned the chromatography on hydroxyapatite columns of calf thymus nucleoprotein[34], denatured transforming DNA[35], replicative RNA from plant viruses[36], mitochondrial yeast DNA[37], and polypeptides and proteins[38,39]; a theory for the chromatography of rigid macromolecules on hydroxyapatite has been developed in our laboratory[40,41].

This paper will report our results on native DNA from animals, bacteria and viruses. The following two papers will deal with the chromatographic behaviour of denatured DNA and of polyribonucleotides, respectively[42,43].

MATERIALS AND METHODS

Preparation of hydroxyapatite

This was done according to the procedure described by TISELIUS *et al.*[20,44], using the following Merck (Darmstadt, Germany) chemicals: $CaCl_2$, 2382; Na_2HPO_4, 6580; NaH_2PO_4, 6346; KH_2PO_4, 4873; K_2HPO_4, 5101 (the numbers are those of the Merck catalogue). In order to obtain columns allowing a "good" flow rate (see below), it is advisable to be generous in discarding the "fines" after the heating step in NaOH. As a rough indication of how much material should be discarded, it can be said that, in our laboratory, a preparation obtained from 2 l of 0.5 M $CaCl_2$ and 2 l of 0.5 M Na_2HPO_4 has a packed volume of 250–300 ml. In the most recent work, we have used a multichannel peristaltic pump (Desaga, Heidelberg, Germany) to feed, at a flow rate of 250 ml/h, the $CaCl_2$ and Na_2HPO_4 solutions into a 5-l beaker containing 200 ml M NaCl. Hydroxyapatite can be stored in 0.001 M sodium phosphate buffer pH 6.8 at 4° for several weeks without any change in its chromatographic properties. Care should be taken not to use a vigorous agitation to re-suspend the hydroxyapatite crystals since this may break them down and slow columns may result.

Other methods for preparing hydroxyapatite have been described by ANACKER AND STOY[45], MAIN, WILKINS AND COLE[24], JENKINS[46] and SIEGELMAN *et al.*[47,48]. We have, however, no experience with these preparations, nor with the commercial products sold by Bio-Rad Laboratories (Richmond, Calif.) and Clarkson Chemical Company (Williamsport, Pa.).

Column chromatography

Packing of the columns was done by adding an hydroxyapatite suspension in 0.001 M phosphate buffer to columns partially filled with the same buffer; the column outlet was open only after a 1-cm layer of hydroxyapatite had settled on the quartz wool plug or the sintered glass filter (No. 2) forming the bottom of the column. Alternatively, columns may be prepared by adding the hydroxyapatite suspension to a

funnel mounted on the top of the column, filled with the starting buffer; the hydroxyapatite suspension in the funnel was mechanically stirred during the preparation of the column. This procedure, suggested by a Pharmacia (Uppsala, Sweden) leaflet describing preparation of Sephadex columns, allows very homogeneous packing.

Elution was usually carried out with potassium phosphate buffer pH 6.8. Sodium phosphate buffer pH 6.8 may be used as well at room temperature, or even in the cold (4°) provided that the molarity is not higher than 0.3 M. The buffer molarity was increased either stepwise or continuously; in this second case, a linear molarity gradient was produced using a Varigrad (Technicon, Chauncey, N.Y.). The phosphate concentration of eluates was checked by phosphorus analysis according to ALLEN[49], or by a refractive index measurement, using a Zeiss (Oberkochen, Germany) instrument of the Abbe type. The ultraviolet transmission of the column effluent, as well as its radioactivity in some experiments, were continuously recorded. Fractions were collected on a volume basis, using in most cases Gilson Medical Electronics (Madison, Wisc.) and LKB (Stockholm, Sweden) fraction collectors.

Routine chromatography experiments were performed at room temperature, generally using 1.3 cm × 7 cm or 1 cm × 10 cm columns and loads of 1 mg of the substance under investigation. If gradient elution was used, the gradient was produced by mixing 100 ml of each 0.001 M potassium phosphate buffer and 0.5 M potassium phosphate buffer. The flow rate obtained under these experimental conditions was 20–40 ml/h using hydrostatic heads of 30–50 cm. The slope of the phosphate gradient reduced to column surface equal to unity, $(\Delta M/\Delta V)\cdot s$ (M being the phosphate molarity, V the volume of eluent and s the surface of the column) was close to 2 mM/cm.

Polynucleotide samples were loaded at widely differing concentrations, ranging from 1 μg to 1 mg/ml; in most cases, solvents were 0.001 M potassium phosphate buffer, 0.01 M potassium phosphate buffer, 0.15 M NaCl; 1 M NaCl and 1 M KCl have also been used. Calcium-complexing agents, like citrate and EDTA, should be absent from the solvents of nucleic acids to be chromatographed. Chloroform–isoamyl alcohol, phenol, formaldehyde, urea may be present in the starting solution.

To check the properties of materials irreversibly retained by the columns (*i.e.* not elutable by 0.5 M or 1 M potassium phosphate buffer), hydroxyapatite was extruded and dissolved by dialysis against 1 M EDTA, pH 8.0. An alternative procedure, used in order to elute denatured DNA irreversibly adsorbed by hydroxyapatite, involved treating the top layers of the column with 0.1 M NaOH. If a simple check on the recovery of radioactive material was desired, the columns were simply dissolved in 1 M HCl, and the solution counted. If it is desired to regenerate hydroxyapatite columns, the top layers are removed and the columns are washed with 0.5 M, or 1 M potassium phosphate buffer, and with 0.001 M potassium phosphate buffer, in succession; alternatively hydroxyapatite is extruded from the columns and boiled for 15 min in 0.001 M potassium phosphate buffer. Regenerated hydroxyapatite was not used in any of the experiments described here.

Materials

Calf thymus DNA and chicken erythrocyte DNA were prepared according to procedures described elsewhere[50]. *Escherichia coli* DNA and *Haemophilus influenzae* DNA were obtained according to LUZZATI, LUZZATI AND MASSON[51] and BACH, LUZZATI

AND CHEVALLIER[52], respectively. DNA's from phages T1, T2, T4, T5, λ and ΦX_{174} were obtained by phenol treatment of the purified phage suspensions. RNA's from 3 plant viruses (tobacco mosaic virus, turnip yellow mosaic virus, alfalfa mosaic virus) were obtained by phenol treatment of the purified virus suspensions. Ribosomal RNA from Ehrlich ascites-tumor cells, *Escherichia coli*, or yeast, soluble RNA from yeast, RNA "core" were preparations described elsewhere[50]. Biosynthesic polyribonucleotides were commercial products purchased from Miles (Clifton, N.J.) or Calbiochem (Los Angeles, Calif.). Nucleic acid derivatives (bases, nucleosides, nucleotides), nucleoside polyphosphates, vitamins and coenzymes were commercial products obtained from Calbiochem, or Pabst (Milwaukee, Wisc.).

Methods

Heat-denaturation of DNA. This was done by immersing erlenmeyer flasks containing the DNA solutions (50–100 μg/ml in 0.13 M NaCl–0.01 M potassium phosphate buffer) in a boiling-water bath for 15 min; solutions were then rapidly cooled by pouring them in flasks kept in an ice bath. In some cases, heat denaturation of DNA was carried out according to the same procedure except that temperatures in the melting range of DNA were used. Boiling water–ethanol mixtures were used to thermostate precisely the DNA solutions in the melting range[54].

Alkaline denaturation of DNA was performed by titrating DNA solutions, at the concentrations indicated above, to pH 12.5. Neutralization was done with 0.5 M KH_2PO_4 or 1 M potassium phosphate buffer. Occasionally, denaturation by electrolyte dilution was used.

Formaldehyde treatment of denatured DNA was done by reacting it with freshly neutralized 1 % formaldehyde (final concentration) at 25° for 24 h.

Ultraviolet melting curves were determined by using a heating cell holder built by Dr. J. POUYET of this laboratory; the temperature within the cell was measured by a thermocouple immerged in the hollow, silicone-containing, glass stopper of the cell.

Other physical properties of the polynucleotides used were determined by using methods and techniques described elsewhere[50,55].

Methylated serum albumin–kieselguhr columns were prepared and used as described by MANDELL AND HERSHEY[4].

RESULTS

Properties of native DNA after chromatography

A series of experiments was aimed at verifying whether any changes in the physical, chemical and biological properties of native DNA take place upon the adsorption–elution process. This preliminary and basic question had been left unanswered by earlier work. In order to solve this problem, hydroxyapatite columns were loaded with DNA, washed with low-molarity potassium phosphate buffer (usually 0.005 M), and then eluted with 0.5 M potassium phosphate buffer. This type of experiment was also used to obtain precise information about the recovery from the columns.

DNA samples from calf thymus, chicken erythrocytes and *E. coli*, displaying molecular weights (as determined by light-scattering) in the $4 \cdot 10^6$–$6 \cdot 10^6$ mol. wt. range, did not show, after the adsorption–elution process, any significant differences with respect to the original samples in any of the following properties: light-scattering envelope (and therefore weight-average molecular weight and radius of gyration), sedimentation coefficient, ultraviolet spectrum, and ultraviolet melting curve. DNA samples from the same sources, but showing a mol. wt. higher than $6 \cdot 10^6$ often showed a lower molecular weight after the adsorption–elution process. This phenomenon was apparently due, in some cases, to the removal of a small amount of large aggregates from the DNA sample; in other cases, in which aggregated samples were prepared by using steps which lead to an aggregation of DNA (*e.g.*, alcohol precipitation at an early stage of the deproteinization procedure) the adsorption–elution process caused a disaggregation of the intermolecular complexes possibly linked through protein material. Interestingly enough, chloroform–isoamyl alcohol (5:1, v/v) treatment of these aggregated DNA samples was much less effective than chromatography on hydroxyapatite in disaggregating the DNA. An interesting observation made in the course of these experiments was that DNA was eluted from hydroxyapatite columns in a dust-free condition as judged by light-scattering.

Results obtained with DNA samples from phages T2, T1, T5 and λ showed that the sedimentation constants of the loaded and the eluted samples were identical. In the case of T2 DNA, preparations of "whole" molecules, as obtained by chromatography on methylated serum albumin–kieselguhr columns, were used. It appears, therefore, that breakage by shearing does not occur during the elution of very high molecular weight DNA from hydroxyapatite.

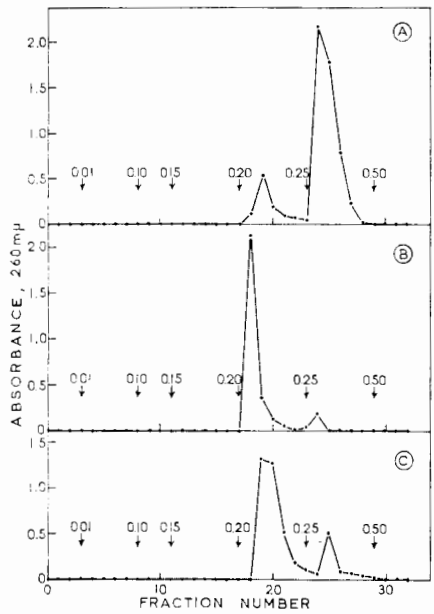
Transforming *H. influenzae* DNA was adsorbed on, and eluted from, hydroxyapatite without any modification in the biological activity of 3 different genetic markers[31,35]. The ultraviolet monitoring system was not used in these experiments, since it caused a conspicuous inactivation of the transforming activity.

In all cases investigated so far, recovery of native DNA from the columns was higher than 90 %, as judged from the loaded and eluted adsorbance at 260 mμ; in the large majority of experiments a complete recovery was obtained.

Chromatographic behaviour of native DNA

This was studied by using both stepwise and gradient elution. When elution was carried out stepwise according to the scheme shown in Fig. 1, all DNA samples tested so far were eluted in 2 fractions, at 0.20 M and 0.25 M potassium phosphate buffer, respectively (Fig. 1A). Occasionally, minor additional fractions were eluted when the phosphate molarity was further raised to 0.30 M and 0.50 M potassium phosphate buffer. Upon re-chromatography, the two fractions and the occasional minor peaks were eluted again in two peaks, at 0.20 M and 0.25 M potassium phosphate buffer, respectively (Figs. 1B, 1C). The relative importance of the two peaks was, as in the first chromatography, determined by the length of the column, the first peak being larger than the second one with short columns, and *vice versa* (Fig. 1). These experiments were carried out with DNA samples from calf thymus or chicken erythrocytes.

When elution was performed with a linear molarity gradient of potassium



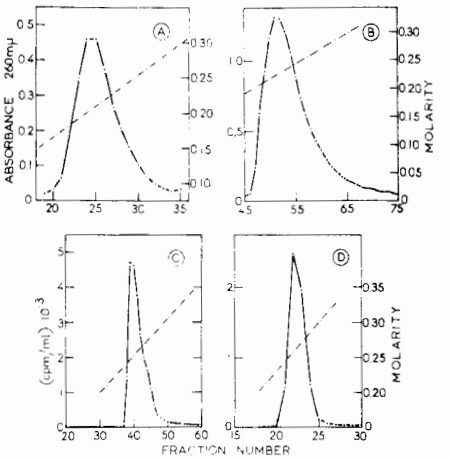


Fig. 1. Chromatography of native calf thymus DNA (preparation A 1). A. Chromatography of 1.28 mg of DNA on a 1.3 cm × 5 cm column. B. Rechromatography of the 0.20 M fraction; 0.56 mg of DNA were loaded on a 1.3 cm × 3 cm column. C. Rechromatography of the 0.25 M fraction; 0.78 mg of DNA were loaded on a 1.3 cm × 3 cm column. In all cases, 3-ml fractions were collected. Rechromatography experiments were done on pooled 0.20 and 0.25 M fractions from two chromatographic experiments. The DNA loading took place at Fraction 0; the stepwise increase in potassium phosphate buffer molarity are indicated by the vertical arrows.

Fig. 2. A. Chromatography of native calf thymus DNA (preparation B 15). 2 ml of a solution having an $A_{260\ m\mu} = 5.0$ were loaded on a 1 cm × 4.5 cm column of hydroxyapatite. This experiment was carried out at 4°. Fractions of about 3.8 ml were collected. Elution was carried out with 100 + 100 ml of 0.001 and 0.5 M potassium phosphate buffer. B. Chromatography of *H. influenzae* DNA. 4 ml of a solution having an $A_{260\ m\mu} = 5.01$ were loaded on a 1 cm × 10 cm column. Fractions of 3 ml were collected. Elution was carried out with a molarity gradient (150 + 150 ml) of potassium phosphate buffer (0.001–0.5 M). Flow rate, 36 ml/h. Recovery was 92 %. C. Chromatography of ^{32}P-labelled T5 DNA on a 1 cm × 6 cm hydroxyapatite column. Elution was carried out with a molarity gradient of potassium phosphate buffer. 2.6-ml fractions were collected. $1.2 \cdot 10^5$ counts/min were loaded and recovered from the column. D. Chromatography of ^{32}P-labelled T2 DNA on a 1 cm × 10 cm hydroxyapatite column. Elution was carried out with a molarity gradient of potassium phosphate buffer containing 1 % formaldehyde. 5.2-ml fractions were collected. Recovery was 92 %. Flow rate, 40 ml/h.

phosphate buffer rather than stepwise, the elution pattern showed only one peak centered at 0.20 M–0.22 M for the DNA samples of $4 \cdot 10^6$–$6 \cdot 10^6$ mol. wt. from higher organisms or bacteria (Figs. 2A, 2B), whereas samples from T5 and T2 phages were eluted at 0.27 M (Figs. 2C, 2D).

DNA preparations from bacteria frequently showed contaminating RNA fractions eluting in the 0.10–0.15 potassium phosphate buffer molarity range, and oligonucleotides (derived from RNA) eluting at very low molarities (around 0.01 M potassium phosphate buffer).

No significant difference was found in the elution molarity of native DNA, whether the experiments were carried out at 4° or at room temperature.

Gradient elution was also done at constant ionic strength, using gradients formed by 0.001 M potassium phosphate buffer + 1 M KCl as the starting buffer and 0.5 M potassium phosphate buffer as the final buffer: in both these solvents the ionic strength was equal to 1.0. In this case, DNA was eluted at the same phosphate molarity as in the absence of KCl (Fig. 3A). If a still higher ionic strength was used, the two buffers being, for instance, 0.001 M potassium phosphate buffer *plus* 2 M KCl and 0.5 M potassium phosphate buffer *plus* 1 M KCl, the phosphate eluting molarity was higher, and equal to 0.27 M. This phenomenon, probably due to the repression of the phosphate ionization, was observed at the 1 M level when using a Na^+ system: for instance, using 0.001 M sodium phosphate buffer+1 M NaCl and 0.5 M sodium phosphate buffer as the limiting buffers, DNA was eluted at a phosphate molarity of 0.31 M (Fig. 3B).

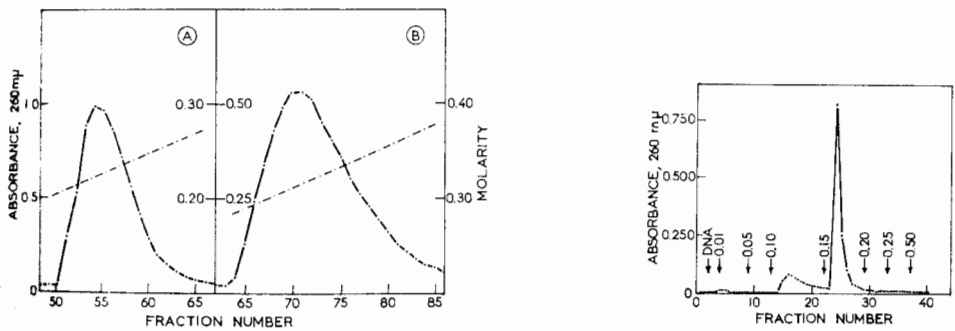


Fig. 3. A. Chromatography of native calf thymus DNA (preparation B 3). 19 ml of a DNA solution having an $A_{260\ m\mu} = 1.38$ were loaded in a 1.3 cm × 7 cm hydroxyapatite column. Elution was carried out with a linear gradient, the limiting solvents being respectively 200 ml potassium phosphate buffer 0.001 M + 1 M KCl and 200 ml 0.5 M potassium phosphate buffer. Fractions of 3.1 ml were collected. The phosphate molarity was determined by phosphorus analysis. Yield 95 %. B. Chromatography of native calf thymus DNA (preparation B 3). 19 ml of a DNA solution having an $A_{260\ m\mu} = 1.19$ was loaded on a 1.3 cm × 7 cm hydroxyapatite column. Elution as carried out with a linear gradient, the limiting solvents being, respectively, 200 ml sodium phosphate buffer 0.001 M + 1 M NaCl and 200 ml 0.5 M sodium phosphate buffer. Fractions of 3.1 ml were collected. The phosphate molarity was determined by phosphorus analysis. Yield, 95 %. Left hand, inner ordinate shows the absorbance.

Fig. 4. Chromatography of ΦX174 DNA on a 1.3 cm × 3 cm hydroxyapatite column; 5 ml of DNA solution having an $A_{260\ m\mu} = 1.68$ were loaded; 3.3-ml fractions were collected. Recovery was 96 %.

Some native DNA's show a chromatographic behaviour which is different from that just reported for native, double-stranded DNA: (a) single-stranded DNA from ΦX174 phage was eluted, using the stepwise elution, in two fractions at 0.10 M and 0.15 M potassium phosphate (Fig. 4; see following papers[42,43] for comments); (b) the twisted circular form of polyoma virus DNA is eluted at a lower molarity than the linear open and circular forms[56]; (c) mitochondrial yeast DNA is eluted at a higher molarity than nuclear DNA[37]; this and the previous case are discussed elsewhere[37,43]; (d) glucosylated DNA from T even phage (see below and DISCUSSION).

Fractionation of native DNA

The re-chromatography experiments quoted above and the results obtained

with the gradient elution suggest that the peaks obtained when using the stepwise elution technique are "false peaks", an artifact already described, in the case of protein chromatography on hydroxyapatite, by TISELIUS, HJERTEN AND LEVIN[20] and by HJERTEN[57]. This interpretation would suggest that hydroxyapatite columns do not fractionate native DNA, at least at the level of chromatographic resolution used in the present work. In this connection, some clearly negative results have been obtained. For instance, no fractionation with respect to base composition has been found with native DNA whether using the stepwise or the gradient elution; this finding confirms similar results by SEMENZA[21]. Similarly, no fractionation of genetic markers has been obtained with native DNA[35].

It remains to be seen whether differences may be found in the physical properties of the fractions obtained from native DNA. This point was investigated using several different experimental approaches. (a) When DNA samples having mol. wts. lower than $6 \cdot 10^6$ were loaded at a low (0.01 M) potassium phosphate buffer molarity and elution was done using 2 steps, 0.20 M and 0.50 M potassium phosphate buffer, respectively, the two fractions so obtained did not show any difference in their light-scattering properties. When the starting samples had mol. wts. close to, or higher than, 10^7, the fraction eluted by 0.20 M potassium phosphate buffer showed a lower molecular weight than that shown when the DNA sample was eluted in a single step of 0.50 M potassium phosphate buffer (compare Sample 4 with Samples 2 and 3 in Table I). (b) By running artificial mixtures of intact DNA and of DNA partially degraded by acid deoxyribonuclease (see below), the differences found in the molecular weights of the first and the second fraction were quite conspicuous (Table I); these experiments, like those described under (a) were done on DNA samples from calf thymus or chicken erythrocytes. (c) Some experiments similar to those just described were done using phage DNA's and gradient elution. In one such experiment,

TABLE I

MOLECULAR WEIGHTS OF DNA AND DNA FRACTIONS

B2 DNA was a sample obtained from chicken erythrocytes.

DNA	*Mol. wt.* $\times 10^{-6}$*	*Z***	R_z***	*Observations*
1. B2	8.0†	4.4	3170	Before chromatography
2. B2	7.25†	4.4	3200	As eluted by 0.5 M potassium phosphate buffer
3. B2	7.25†	4.0	2790	
4. B2	5.0	4.1	2300	As eluted by 0.2 M potassium phosphate buffer
5. B2 t	1.4	2.75	1470	Degraded by acid deoxyribonuclease
6. B2 + B2 t(50 : 50)	3.2	3.4	2000	Fraction eluted by 0.2 M potassium phosphate buffer
7. B2 + B2 t(50 : 50)	5.4	3.7	2900	Fraction eluted by 0.5 M potassium phosphate buffer

* All light-scattering measurements were performed using 1 M NaCl, as the solvent.
** Dissymmetry.
*** Radius of gyration.
† The molecular weights of these DNA samples are likely to be underestimated[63].

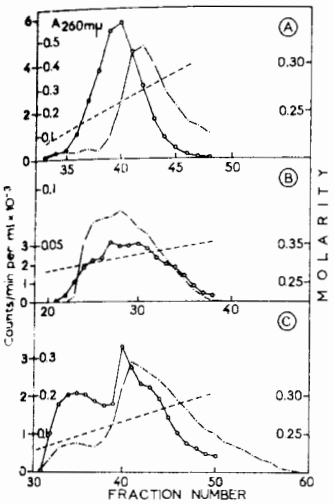


Fig. 5. A. Chromatography of a mixture of sonicated ^{32}P-labelled T2 DNA (0.45 ml; $2.7 \cdot 10^5$ counts/min per ml) and unlabelled T2 DNA "whole" molecules (9 ml; $A_{260\,m\mu} = 1.00$; these were obtained by chromatography of T2 DNA on a methylated serum albumin–kieselguhr column) on a 1 cm × 6 cm hydroxyapatite column. Elution was carried out with a linear molarity gradient of potassium phosphate buffer. About 92 % of both radioactivity and absorbance at 260 mμ was recovered. ○–○, radioactivity measurements; · — ·, absorbance at 260 mμ. Flow rate, 26 ml/h. B. Chromatography of a mixture of ^{32}P-labelled T2 DNA half-molecules and cold T2 whole molecules (obtained by chromatography on methylated albumin–kieselguhr column of sheared T2 DNA and T2 DNA, respectively) on a 1 cm × 8 cm hydroxyapatite column. Elution was carried out with a linear molarity (0.20–0.40 M) of potassium phosphate buffer. 86 % of both radioactivity and absorbance at 260 mμ was recovered. ○–○, radioactivity measurements; · — ·, absorbance at 260 mμ. C. Chromatography of a mixture of ^{32}P-labelled T5 DNA and cold T2 DNA (whole molecules as obtained by chromatography on methylated albumin–kieselguhr columns) on a 1 cm × 12 cm hydroxyapatite column. Elution was carried out with a molarity gradient (0.20–0.40 M) of potassium phosphate buffer. ○–○, radioactivity measurements; · — ·, absorbance at 260 mμ.

a mixture of unlabelled T2 phage DNA "whole" molecules (mol. wt. approx. $1.3 \cdot 10^8$; refs. 58, 59) could be separated to a fair extent from sonicated ^{32}P-labelled T2 DNA molecules (mol. wt. approx. $5 \cdot 10^5$) (Fig. 5A), but not at all from T2 DNA "half" molecules (Fig. 5B); these were obtained by shearing, and separated from "whole" molecules by chromatography on methylated albumin columns. (d) The eluting molarities of calf thymus DNA samples ranging in mol. wt. from $6 \cdot 10^6$–$1 \cdot 10^5$ were compared, using the linear gradient technique. These samples were obtained by limited degradation with acid deoxyribonuclease, an enzyme known to break both DNA strands at the same level[50,60] and to cause no significant change in the secondary structure of DNA in the mol. wt. range under consideration. All the samples analysed were eluted at the same phosphate molarity of 0.20–0.22. Similar results were obtained by DNA samples sheared in a high-speed Vir-Tis homogenizer in the presence of chloroform–isoamyl alcohol (5:1, v/v) (E. G. RICHARDS, unpublished experiments, 1962). In contrast to this behaviour, DNA samples from bacteria or higher animals displaying molecular weights in excess of 10^7 often were eluted at a slightly higher molarity (0.22–0.25). As already mentioned, the large DNA's from T2 and T5 phages were eluted at about 0.27 M potassium phosphate buffer. (e) Glucosylation of T2 DNA

seems to affect to some degree the chromatographic behaviour of this DNA. In fact, a mixture of unlabelled T2 DNA and of ^{32}P-labelled T5 DNA could be resolved to some extent (Fig. 5C), whereas the T2 "half" molecules, which have a mol. wt. close to that of T5 could not be separated (Fig. 5B).

DISCUSSION

The results obtained in the investigations of DNA as eluted from hydroxyapatite columns clearly indicate that no significant changes in the physical, chemical or biological properties take place upon the adsorption–elution process. The recovery of native DNA from the columns has been found to be complete in the vast majority of the cases; incomplete recoveries were obtained only when DNA samples had been poorly deproteinized and/or contained aggregated material. As mentioned above, this was sometimes disaggregated by the adsorption–elution procedure.

The chromatographic behaviour of all native DNA samples studied so far is characterised by the fact that they are eluted in a single peak by a potassium phosphate buffer molarity gradient; the eluting molarity for DNA preparations from bacteria and higher animals is 0.20–0.22; that for high-molecular weight viral DNA's is higher (0.25–0.27). This latter phenomenon may be due to a retardation of these enormous molecules with respect to the front of the eluting buffer (see also below).

When using the stepwise elution technique, native DNA is eluted in two peaks, at 0.20 M and 0.25 M potassium phosphate buffer, respectively; the relative proportions of these two fractions depend upon the ratio DNA load to hydroxyapatite bed. The load being the same, the first fraction is larger than the second one when using small columns, whereas the opposite is true with large columns. Since, upon re-chromatography, these fractions show the same elution pattern as the starting DNA, these peaks may be considered as "false peaks" (refs. 20, 57). This behaviour is due to the fact that 0.20 M potassium phosphate buffer is a solvent just able to start the elution of DNA and therefore the amount of DNA which is eluted will depend very critically upon the column bed to load ratio used.

As far as the fractionation of native DNA on hydroxyapatite columns is concerned, definitely negative results have been obtained with respect to the possibility of separating fractions having different base composition or carrying different genetic markers. The columns seem also to be highly insensitive to differences in molecular lengths since no differences were found in the eluting potassium phosphate buffer molarities of DNA samples ranging in mol. wt. from $6 \cdot 10^6$ to $1 \cdot 10^5$. In contrast, moderate degrees of fractionation according to the molecular size were clearly obtained when using artificial mixtures of fragmented and intact DNA. In these cases two different factors may be thought to play a role in the fractionation: (a) there may be a displacement of the smaller molecules by the larger ones; upon the elution process the first ones would be desorbed first; this may explain such facts as the fractionation shown in Table I; (b) a physical fractionation according to size may occur during the flow of the eluted DNA through the column and be superimposed on the previous mechanism; this fractionation according to the hydrodynamic volume of the DNA molecules possibly also explains the partial separations of glucosylated and unglucosylated phage DNA molecules. Both this non-chromatographic type of fractionation and the displacement effects quoted above should be investigated in more detail.

A general application of chomatography of native DNA on hydroxyapatite, which deserves to be mentioned here because of its practical importance, concerns the characterization and purification of DNA. Indeed, a single, simple experiment like the gradient elution of a DNA sample from a hydroxyapatite column can give very useful information, which would be difficult to obtain otherwise, regarding the presence of denatured DNA, RNA, oligonucleotides, proteins polysaccharides and small organic molecules, and at the same time, purifies DNA from these contaminants. In fact, as it will be shown in the following two articles[42],[43] denatured DNA and RNA are eluted at phosphate molarities which are distinctly lower (0.10–0.15 M) than those eluting native, double-stranded DNA; oligonucleotides are eluted at even lower molarities (0.001–0.015 M); most proteins are also adsorbed less strongly than native DNA; polysaccharides and small organic molecules are adsorbed very weakly, if at all. In the case of viral DNA (and RNA), the technique may be very useful in the preparation of nucleic acids, since phenol-treated viruses may be loaded as such on hydroxyapatite columns and nucleic acids may be eluted free from denatured protein, after the phenol has been washed through with the initial buffer[32].

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