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# CHROMATOGRAPHY OF NUCLEIC ACIDS ON HYDROXYAPATITE

## II. CHROMATOGRAPHY OF DENATURED DNA

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SUMMARY

The chromatographic behavior of DNA denatured by heat at various temperatures, and reacted or not with formaldehyde, on hydroxyapatite columns has been investigated using both stepwise and gradient elution. The recovery of denatured DNA from the columns and the behavior of the chromatographic fractions upon rechromatography have been studied.

The bulk of denatured DNA from bacteria and higher animals is eluted from hydroxyapatite columns by a phosphate molarity distinctly lower than that eluting native DNA, whereas a small "native-like" fraction is eluted at the same molarity as native, renatured, and denatured, cross-linked DNA. Investigations on the ultraviolet absorbance–temperature profile and formaldehyde reaction of the "native-like" fraction showed that it is endowed, to a large extent, with complementary base pairing. Subsequent work has shown that the "native-like" fraction is the carrier of the residual transforming activity surviving denaturation, in the case of bacterial transforming DNA; whereas, it is formed by satellite DNA in the case of DNA's from higher animals.

Denatured, single-stranded DNA molecules may be fractionated on hydroxyapatite columns according to their base composition.

## INTRODUCTION

The behavior of native DNA from viruses, bacteria and higher organisms on hydroxyapatite has been described in the preceding paper[1]. It has been shown that hydroxyapatite columns provide a new, powerful tool for characterizing, purifying and preparing native DNA.

Investigations on the chromatographic behavior of denatured DNA on hydroxyapatite columns showed that this is quite different from that of native DNA; the bulk of denatured DNA is eluted by molarities of potassium phosphate buffer, pH 6.8 which are distinctly lower than those eluting native molecules. In fact, these investigations have provided the first indication that the discriminating power of the columns is very highly sensitive to the secondary structure of nucleic acids. The ability of distinguishing differences in the secondary structures of nucleic acids, first recognized in this laboratory several years ago[2], is the fundamental property of

hydroxyapatite columns; this is accompanied by a high degree of insensitivity to the molecular size of nucleic acids. A presentation of the results obtained with denatured DNA is given in this paper; preliminary reports have been published elsewhere[2,3].

The main finding of the present work is the demonstration of a small "native-like" fraction in denatured DNA from bacteria and higher organisms. In contrast with the bulk of denatured DNA, which is eluted at a phosphate molarity distinctly lower than that of native DNA, this fraction is eluted at the same molarity as native, renatured, and denatured cross-linked DNA. This fraction is endowed with complementary base pairing, at least to a large extent, and is the carrier of the residual transforming activity surviving denaturation in the case of bacterial DNA[4,5], whereas in the case of higher organisms it is formed by satellite DNA[6] and, presumably, by mitochondrial DNA, if this is present.

Another finding of this work is that single-stranded, denatured DNA molecules can be fractionated on hydroxyapatite columns according to their average base composition; this fractionation might be due to slight differences, related to base sequence, in the secondary structure of denatured DNA.

## MATERIAL AND METHODS

These have been described in the preceding paper[1].

## RESULTS

*Chromatographic behavior of denatured DNA: stepwise elution*

The chromatographic behavior obtained with denatured DNA using the stepwise elution method was investigated on DNA samples from calf thymus or chicken erythrocytes which had been subjected to different treatments: (a) heat denaturation at 100°, followed by fast-cooling; (b) heat denaturation at different temperatures between 85° and 100°, followed by fast-cooling and reaction with formaldehyde; (d) heat denaturation at 100° in the presence of 1 % formaldehyde, followed by fast-cooling. In all cases, the properties of the fractions obtained were investigated by submitting them to re-chromatography experiments.

Using the stepwise elution technique, heat-denatured DNA showed three equivalent fractions which were eluted at 0.15 M, 0.20 M and 0.25 M potassium phosphate buffer, respectively (Fig. 1C); minor fractions were occasionally eluted at 0.10 M and 0.50 M potassium phosphate buffer. This behavior is remarkably different from that shown by native DNA which, when run under identical experimental conditions, was eluted, as already indicated in the preceding paper[1], in two fractions, a smaller one eluting at 0.20 M and a larger one eluting at 0.25 M potassium phosphate buffer, occasionally followed by a minor fraction eluted by 0.30 M or 0.50 M potassium phosphate buffer (Fig. 1A). When DNA was heated up to temperatures comprised between 85° and 100°, the elution patterns obtained were intermediate between those of native and heat-denatured (100°) DNA; as increasing temperatures were used for denaturing DNA, increasing amounts of material were eluted at lower molarities; in other words, a gradual shift to the left of the chromatogram was obtained when running DNA samples which had been heated up to increasing tempera-

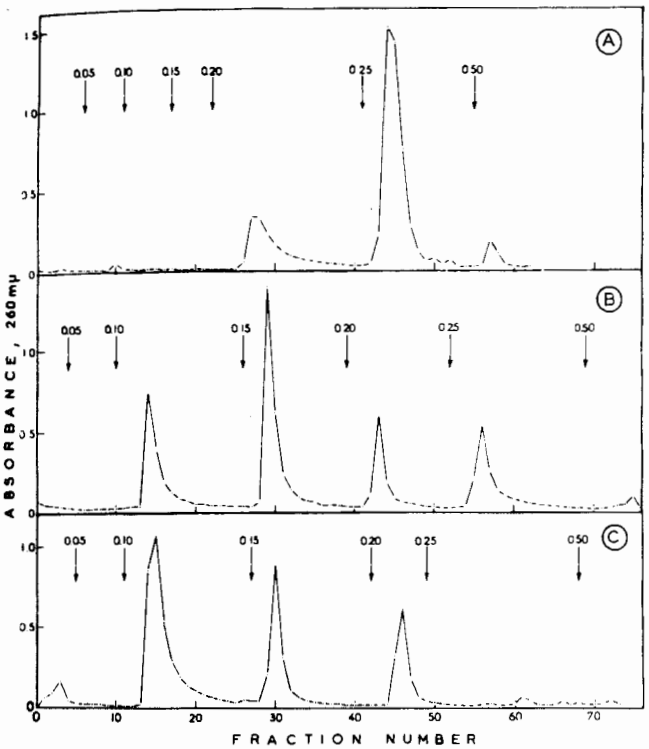



Fig. 1. Chromatography of calf thymus DNA (preparation B 3) on 1.3 cm × 7 cm hydroxyapatite columns. 10–20 ml DNA solutions, having an $A_{260\ m\mu}$ in the 1–2.5 range, were loaded at fraction number zero. 3.8-ml fractions were collected. Recoveries were 100 per cent, except where otherwise stated. Stepwise elution of: (A) native DNA; (B) DNA heated up to 90° and then fast-cooled; (C) DNA heated up to 100° and then fast-cooled; in this case the recovery was 95 %. (Reproduced from *Nature*, 206 (1965) 779.)

tures in the range 85–100°. Fig. 1B shows the chromatogram obtained with a DNA sample heated up to 90°.

The behavior of heat-denatured, formaldehyde-reacted DNA was studied using eluting buffers which contained 1 % formaldehyde. The chromatographic pattern was slightly different from that just described for heat-denatured DNA, since most of the material was eluted by 0.15 M potassium phosphate buffer and smaller fractions were eluted at 0.10 M, 0.20 M, 0.25 M; occasionally, a minor fraction was eluted at 0.50 M (Fig. 2B). The elution profile appeared therefore shifted to the left when compared with that obtained when using heat-denatured DNA which had not been reacted with formaldehyde. In contrast, native DNA treated with formaldehyde and eluted with formaldehyde-containing buffers, showed the same elution pattern as native DNA run in the usual conditions (Fig. 2A; compare this figure with Fig. 1A).

DNA which had been heated to 100° in the presence of 1 % formaldehyde (under which conditions the melting temperature is lowered by 10–15°, see ref. 7) showed, upon chromatography with formaldehyde-containing buffers, an elution

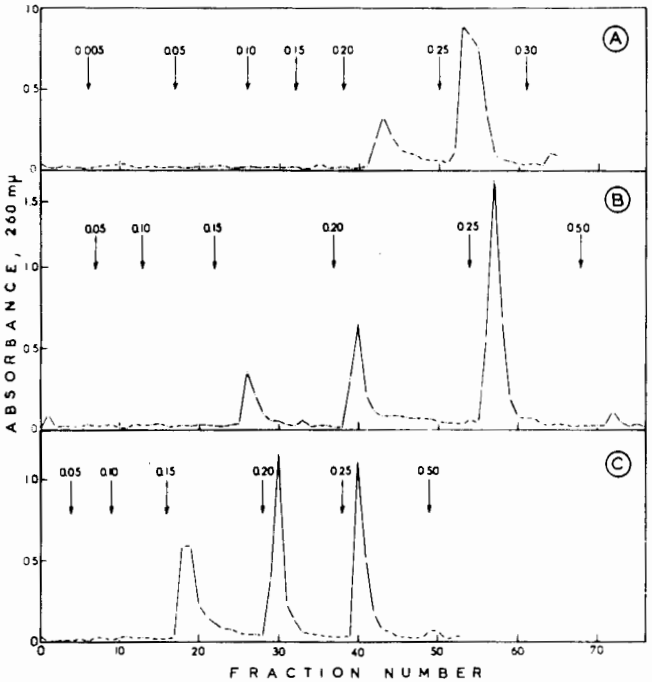
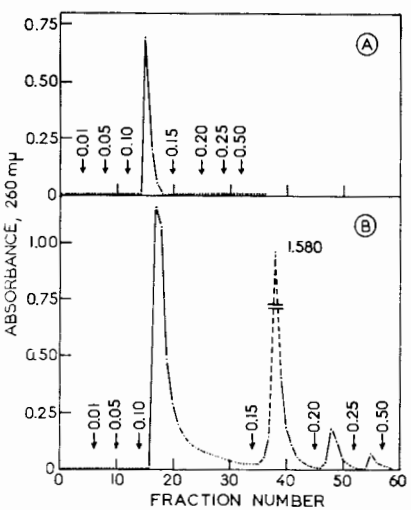



Fig. 2. Stepwise elution in the presence of 1 % formaldehyde, of: (A) native DNA; (B) DNA heated up to 100°, fast-cooled and then reacted with formadehyde; (C) DNA heated up to 100° in the presence of formaldehyde; in this case the recovery was 93 %. For all other indications, see legend of Fig. 1. (Reproduced from *Nature*, 206 (1965) 779.)



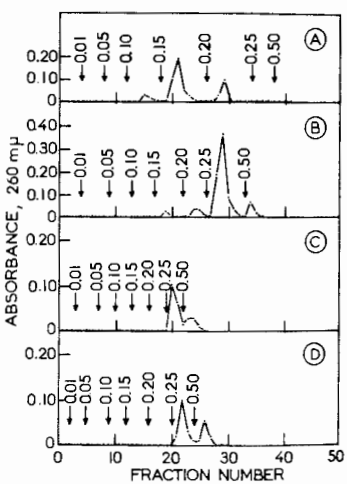




Fig. 3. Re-chromatography experiments performed on the fractions obtained from heat-denatured, fast-cooled and formaldehyde-reacted DNA. Stepwise elution of: (A) the 0.10 M fraction; (B) the 0.15 M fraction.

Fig. 4. Re-chromatography experiments performed on the fractions obtained from heat-denatured, fast-cooled and formaldehyde-reacted DNA. Stepwise elution of: (A) the 0.20 M fraction; (B) the 0.25 M fraction; (C) the 0.25 M fraction from B (third chromatography); (D) the 0.50 M fraction.

pattern further shifted to the left as compared with that of heat-denatured, formaldehyde-treated DNA (Fig. 2C; compare this figure with Fig. 2B). An important feature of the elution profile obtained under these conditions is the absence of fractions eluting at molarities higher than 0.20 M potassium phosphate buffer.

Re-chromatography experiments performed on the fractions obtained from heat-denatured, fast-cooled, formaldehyde-reacted DNA showed the following results: (a) the 0.15 M and 0.10 M fractions contained, respectively, very little and no material eluting at molarities higher than 0.15 M (Figs. 3A, 3B); (b) the 0.20 M fraction showed two main fractions eluting at 0.15 M and 0.20 M, and a minor one eluting at 0.10 M (Fig. 4A); (c) the 0.25 M and 0.50 M fractions contained, respectively, very little and no material eluting at molarities lower than 0.25 M (Figs. 4B, 4D); upon a third chromatography, the 0.25 M fraction did not show any material eluting at a lower molarity (Fig. 4C).

*Chromatographic behavior of denatured DNA: gradient elution*

The re-chromatography experiments just mentioned suggest the existence in heat-denatured DNA of two distinct fractions: a large one eluting at molarities lower than 0.20 M, and a small one eluting at molarities higher than 0.20 M. This conclusion is clearly supported by the gradient elution experiments, as well as by other experiments which will be reported below (see *Properties of the two fractions of denatured DNA*).

Using the gradient elution procedure, heat-denatured, formaldehyde-reacted DNA is eluted in one main peak at about 0.12 M potassium phosphate buffer, followed by a smaller fraction at about 0.20 M (Fig. 7A). These two fractions are equivalent to the DNA fractions eluting below and above 0.20 M, respectively, in the stepwise chromatography. Their chromatographic validity was demonstrated by re-chromatography.

Chromatographic patterns very similar to that shown in Fig. 7A can be obtained using heat-denatured or alkali-denatured DNA not reacted with formaldehyde (Fig. 7A, see also Figs. 3 and 4 of ref. 5); the only difference in the patterns shown by denatured DNA which had not reacted with formaldehyde was that the amount of material eluted at about 0.20 M was larger than in the case of formaldehyde-reacted DNA. This finding parallels the results obtained with the stepwise elution technique (compare Figs. 1C and 2B).

Because of the different elution molarities of native and heat-denatured DNA, an artificial mixture of these can be easily separated by gradient elution except for the small fraction of heat-denatured DNA eluted at about 0.20 M potassium phosphate buffer, which is removed from the column together with native DNA (see Fig. 3 of ref. 3).

*Recovery of denatured DNA from the columns*

The recovery from the columns of heat or alkali-denatured DNA was often found to be incomplete and yields of only 50–80 % were not rare. This is particularly true for large viral DNA's from T2 and T5 phages, in which cases yields of 50 % were the best ones obtained. The following findings suggest that low recoveries may be due

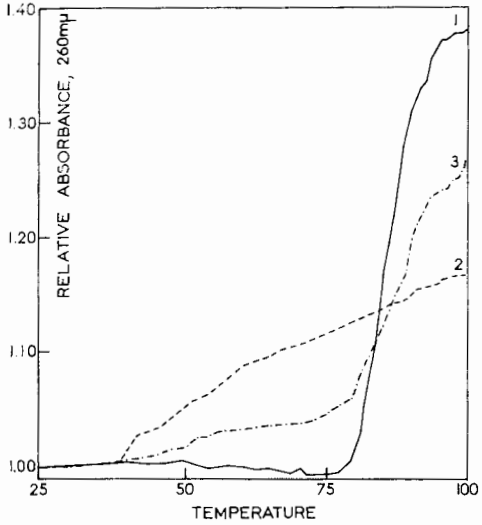
to aggregation of denatured DNA molecules, mediated by residual protein, and/or non-specific intermolecular base pairing: (a) Repeated deproteinization treatments of native DNA samples from bacteria or higher organisms with chloroform–isoamyl alcohol improves the recovery from the columns of these samples after they have been denatured; since this treatment shears DNA, while deproteinizing it, it is impossible to decide from this type of experiment whether a decrease in molecular weight or deproteinization, or both, are responsible for the better yields. (b) Treatment of denatured DNA with formaldehyde improves the recovery; this could be due to the fact that formaldehyde prevents non-specific base pairing as well as interactions between the phosphate groups of DNA and the amino groups of contaminating protein. (c) Heat denaturation of DNA in the presence of formaldehyde gives, as a rule, complete recoveries, probably because of the blocking of all amino groups. (d) In the case of DNA samples from bacteria and higher organisms, it has been observed that low recoveries predominantly affect the first large fraction and not the second small one, so that when recovery is particularly low, the second fraction may become as important as the first one. This point was checked by eluting the irreversibly adsorbed fraction with NaOH or EDTA. This fraction was shown to have a melting curve similar to that of the first fraction (see below). When it was emulsified with chloroform–isoamyl alcohol and rechromatographed, it eluted mainly at the position of the first fraction. (e) Raising the ionic strength up to 2–3 before cooling (or neutralizing if alkali denaturation was used) raises the recoveries of denatured DNA to over 90 %. This method was inspired by the conditions used by FAULHABER AND BERNARDI[8] to dissociate calf-thymus nucleoprotein and has been widely used in recent work[6].

*Properties of the two fractions of denatured DNA*

The two fractions just described have been found in all DNA samples from both bacterial and animal sources investigated so far in this laboratory. They have been studied in respect to their secondary structure, and, in the case of transforming *H. influenzae* DNA, their biological activities[4].

In order to estimate the secondary structure of the chromatographic fractions obtained by stepwise elution from heat-denatured and formaldehyde-reacted DNA from calf-thymus and chicken erythrocytes, these fractions were dialyzed against 0.13 M NaCl–0.01 M potassium phosphate buffer and their melting curves determined. In contrast with the 0.10 and 0.15 M fractions, which exhibited a continuous slow increase in $A_{260\,m\mu}$ in the 40–100° temperature range, the 0.25 M fraction showed a sharp increase in ultraviolet absorption in the 80–100° range (Fig. 5). Furthermore, the total increase in absorption was about 25 % for the 0.25 M fraction and only 15 % for the 0.15 M fraction. The results obtained with chromatographic fractions from heat-denatured calf thymus and chicken erythrocytes DNA, which had not been reacted with formaldehyde and had been fractionated in the absence of formaldehyde, were similar to those just reported, except that the melting curve of the 0.25 M fraction was less sharp, because the "native-like" fraction was more heavily contaminated with denatured material. Similar results were obtained in the case of denatured *H. influenzae* DNA (see Fig. 6 of ref. 5).

Another method used to estimate the extent of the secondary structure of the chromatographic fractions obtained from heat-denatured, fast-cooled, and formalde-



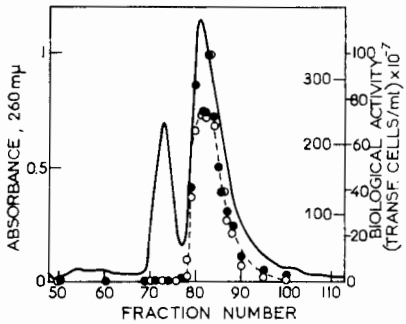




Fig. 5. Melting curves obtained with chromatographic fractions obtained by stepwise elution from heat-denatured, fast-cooled and formadehyde-reacted DNA from calf thymus. The fractions were dialyzed against 0.13 M NaCl–0.01 M potassium phosphate buffer; the melting curves shown refer to: (2) the 0.15 M fraction; (3) the 0.25 M fraction. Curve 1 shows the melting curve of the starting native DNA.

Fig. 6. Chromatography of a mixture of renatured and native DNA. 100 ml of DNA sample N. (20 $\mu$g/ml in 0.15 M NaCl–0.01 M sodium phosphate buffer pH 7.0) were heat-denatured and re-annealed at 67° for 1 h; the sample was loaded on a hydroxyapatite column after addition of 1 mg of native DNA carrying the erythromycin marker. Elution was carried out with a linear molarity gradient (100+100 ml) of potassium phosphate buffer (0.01–0.5 M); 2.7-ml fractions were collected. Recovery of absorbance was 74 %; recovery of biological activity was 74 % for the erythromycin marker (points; inner scale) and 85 % for the cathomycin marker (circles; outer scale). This experiment was carried out by M. R. CHEVALLIER.

hyde-reacted DNA was to dialyze the fractions against 0.13 M NaCl–0.01 M potassium phosphate buffer, containing 1 % formaldehyde (this step having the purpose of having all fractions in the same solvent) and heat them to 100° for 15 min. The increase in absorbance at 260 m$\mu$ caused by the heating step was taken as a measure of the "residual hypochromicity" of the fractions, which is related to the extent of their complementary base pairing. Further comments on this procedure and its meaning are given in the APPENDIX.

The "residual hypochromicities" shown by the chromatographic fractions of heat-denatured, formaldehyde-reacted DNA samples from calf-thymus and chicken erythrocytes were negligible for the 0.10 M and 0.15 M fractions, whereas they ranged from 15 to 25 % for the 0.25 M and 0.50 M fractions. The starting, unfractionated, denatured and formaldehyde-reacted DNA showed a residual hypochromicity close to 5 %, obviously due to the fact that it contains the high eluting, "native-like" fraction. It is interesting to point out that native DNA, when heated to 100° in the presence of formaldehyde, was found to show a hyperchromicity of 38–41 %, definitely higher than the 34 % obtained by reacting heat-denatured, fast-cooled DNA with formaldehyde. This difference can also be explained by the fact that the "native-like" fraction reacts very little with formaldehyde.

Results obtained with alkali-denatured bacterial DNA, not reacted with formal-

dehyde and eluted by a molarity gradient were very similar to those reported above for DNA's from higher animals, in that they also showed that denatured DNA could be fractionated into a large fraction eluting at about 0.15 M phosphate, and a small "native-like" fraction, eluted at about 0.20 M–0.22 M phosphate. In the case of *H. influenzae* DNA the residual transforming activity was found in the second fraction, whereas the first one was practically inactive; a detailed investigation on denatured *H. influenzae* DNA has been presented elsewhere[4].

*Fractionation of denatured DNA*

The finding of different extents of secondary structure in the fractions eluted by increasing molarities of phosphate prompted analytical work on the fractions obtained from calf thymus and chicken erythrocytes DNA's.

Analysis showed that, in both cases, A and T increase, whereas G and C decrease

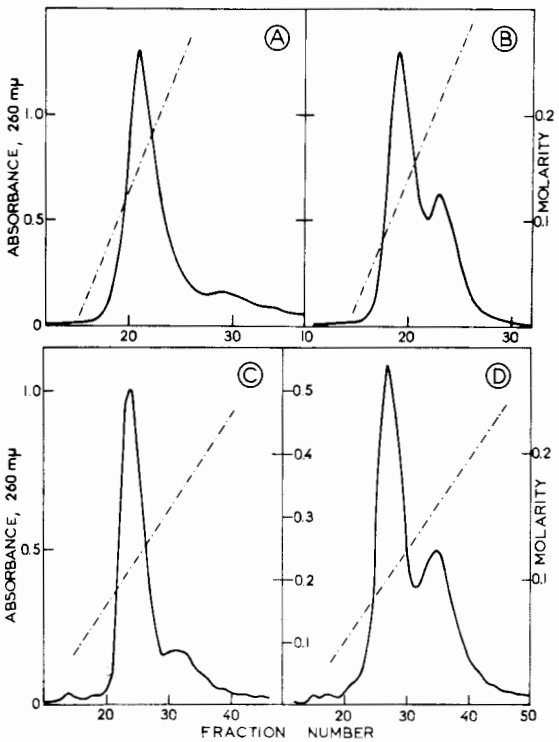



Fig. 7. Chromatography of denatured salmon sperm DNA. A. Intact DNA. B. DNA treated with mustard gas (230 moles/20 000 nucleotides). Both samples were dissolved in $5.10^{-4}$ M potassium phosphate buffer at a concentration of 2 mg/ml; after 48 h at room temperature, the samples were diluted to 50 ml with double-distilled water and kept 30 min at 37°. After cooling to room temperature, samples were made 0.15 M in NaCl by addition of 1 M NaCl. The addition of 1 M NaCl caused a decrease in the $A_{260\ m\mu}$ of the samples of 19 %. Both samples were then treated with 1 % formaldehyde and chromatographed in the presence of formaldehyde. In both runs 27-ml solutions of DNA ($A_{260\ m\mu} = 0.910$ for A; $A_{260\ m\mu} = 0.725$ for B) were loaded in 1 cm × 10 cm columns. 100 ml of each 0.001 M potassium phosphate buffer and 0.5 M potassium phosphate buffer were used for the gradient elution. The experiments shown in C and D also concern intact and alkylated salmon sperm DNA, respectively; the only difference with those reported in A and B is that the formaldehyde treatment was omitted.

in fractions eluted by increasingly higher phosphate molarities. Detailed results will be published elsewhere.

*Chromatography of single-stranded DNA*

As already mentioned in the previous paper, single-stranded DNA from $\Phi$X174 phage was eluted by stepwise elution, in two peaks at 0.10 M and 0.15 M potassium phosphate buffer (see Fig. 5 of the preceding paper[1]. This behavior is rather similar to that of denatured DNA, except that in this case no material is eluted at molarities of 0.20 M or higher.

*Chromatography of renatured DNA*

*H. influenzae* DNA which had been alkali-denatured, neutralized and then renatured, showed (when the gradient elution technique was used) one main peak eluting at the same molarity as native DNA, preceded by a minor fraction eluting at the molarity of denatured DNA (Fig. 6).

*Chromatography of cross-linked DNA*

The chromatographic behavior of cross-linked, denatured DNA was studied by using salmon sperm DNA which had been partially cross-linked by mustard gas. The results obtained by running these samples in the presence and in the absence of formaldehyde, as well as their control samples, are shown in Fig. 7. A much larger amount of DNA was eluted at the position of native DNA in the cross-linked samples compared to the control samples (compare Figs. 7A and 7C with Figs. 7B and 7D); furthermore, running the samples, after reaction with formaldehyde, in formaldehyde-

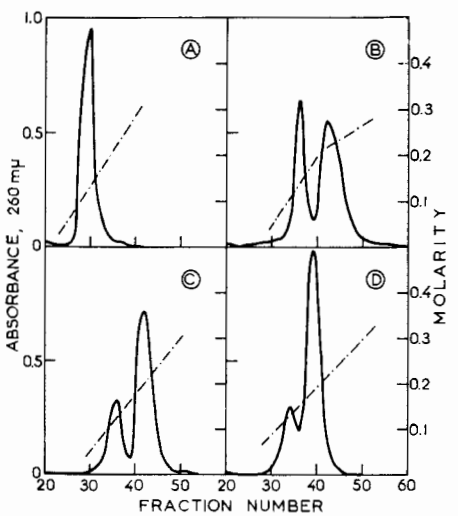



Fig. 8. Chromatography of heat-denatured *Micrococcus lysodeikticus* DNA, treated with nitrous acid for 0(A), 6(B), 12(C) and 60(D) min. Nitrous acid treatment was performed at pH 4.2; samples were then neutralized, dialyzed against 0.15 M NaCl and heated for 10 min at 120°. Recoveries were 77 %(A), 92 %(B), 87 % (C) and 80 % (D), respectively. This experiment was performed by M. L. GRETH.

containing buffers reduced the amount of material eluting at the position of native DNA (compare Figs. 7A and 7B with Figs. 7C and 7D). Experiments in which DNA was cross-linked by nitrous acid and then heat-denatured are reported in Fig. 8. The patterns shown in Figs. 8A, 8B, 8C and 8D refer to *Micrococcus lysodeikticus* DNA samples treated with nitrous acid for 0, 6, 12 and 60 min, respectively. The extremely small amount of "native-like" fraction present in denatured *M. lysodeikticus* DNA (Fig. 8A) seems to be due to the low molecular weight of this DNA (mol. wt. $= 4.8 \cdot 10^6$), as well as to the very high temperature (120°) used to denature it.

## DISCUSSION

Both stepwise and gradient elution results presented in the preceding section clearly show that the bulk of denatured DNA from bacteria and higher animals is eluted at a phosphate molarity which is lower than that of native DNA. The chromatographic behavior of the main fraction of native DNA is very similar to that of single-stranded DNA from $\Phi$X174 phage.

In contrast, a small fraction of denatured DNA shows the chromatographic behavior of double-stranded DNA's, like native DNA, renatured DNA, and denatured cross-linked DNA. This fraction has been called "native-like". Its existence, first shown in this laboratory several years ago[2,3], has been confirmed by the independent work of ALBERTS AND DOTY[9] and MULDER AND DOTY[10], who isolated it by using the aqueous dextran–polyethylene glycol two-phase system of ALBERTSSON[11], and also by WALKER AND MCLAREN[12], who prepared it according to our procedure[2,3] from sonicated mouse DNA.

The two main questions which may be asked about the "native-like" DNA fraction concern its structure and its origin. That the "native-like" fraction is endowed, to a large extent, with complementary base pairing is suggested by the following lines of evidence: (a) Its elution molarity on hydroxyapatite columns is that shown by double-stranded polynucleotides[13]. It should be pointed out, however, that this chromatographic behavior, by itself, is no proof that the "native-like" fraction is double-stranded, since neutral polyadenylic acid, which has a helical, single-stranded structure at neutral pH, is also eluted at about the same molarity as double-stranded polynucleotides[13]. (b) If DNA is heated up to 100° in the presence of formaldehyde, no "native-like" fraction appears in the chromatogram. (c) Its melting profile and its "residual hypochromicity" approach those of native DNA. The existing differences, namely the lower hypochromicity and the increase in $A_{260\,m\mu}$ in the 50–75° temperature range, are, at least to a large extent, due to the contamination of the "native-like" fraction by the single-stranded molecules of the main fraction of denatured DNA. This contamination is due to the fact that the tail of the main fraction overlaps the small peak of the "native-like" fraction in the chromatograms, particularly when chromatograms are run in the absence of formaldehyde. However, experiments by CHEVALLIER AND BERNARDI[5] on *H. influenzae* DNA, and ANDRÉ AND BERNARDI[6] on calf thymus DNA suggest that the "native-like" fraction is slightly different in structure from native DNA, in that its double-stranded structure contains disordered regions. The chromatographic behavior of the "native-like" fraction cannot be distinguished, at least at the resolution level used in the present work, from that

of native DNA probably because hydroxyapatite columns are not very sensitive to the presence of disordered regions in otherwise double-stranded molecules.

A very important question concerns the origin of the "native-like" DNA fraction. Only two explanations will be considered here. The "native-like" fraction might be formed either (a) by DNA molecules, whose strands never came apart and therefore could re-unite in register when DNA was transferred to a non-denaturing environment; this would be the case if a cross-linkage of some sort existed in a small percentage of the DNA molecules; (b) by DNA molecules, whose strands did come apart; in this case "native-like" structures might form by reassociation of the complementary strands of DNA molecules rich in repetitive sequences [14,15]. The first explanation seems to be valid in the case of bacterial DNA[4,5,9,10]. The second one is probably correct in the case of DNA's from animal sources, since it has been shown that in calf-thymus DNA the "native-like" fraction is formed by satellite DNA[6].

A last point which deserves to be commented upon is the fractionation of denatured DNA according to its average base composition, A,T-rich molecules being eluted at lower molarities than G,C-rich molecules. Considerations which are presented in the APPENDIX and the finding that A and T, and G and C, respectively, are not present in equimolar amounts in the chromatographic fractions (work to be published) strongly suggest that hydroxyapatite columns fractionate single-stranded DNA molecules. A possible explanation for this fractionation according to base composition is that single-stranded molecules posses, to some extent, ordered structure due to base stacking (which is, in turn, related to base sequences) and that hydroxyapatite columns discriminate between these slightly different structures. Experiments performed with polyribonucleotides[13] support this interpretation. It should be remarked that the "native-like" fraction of calf-thymus DNA fits by chance the trend of increasing GC content with increasing elution molarity shown by denatured DNA, since it is formed by satellite DNA's having a higher GC content than the main DNA.

This coincidence was one of the reasons which led us to suggest a different interpretation of the chromatogram obtained with denatured DNA[3]; the other reason being that our older analytical results seemed to indicate equimolar amounts of A and T, and G and C, respectively.

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APPENDIX

*Reaction of formaldehyde with calf thymus DNA heated at different temperatures*

The utility of formaldehyde in stabilizing single-stranded DNA molecules and in investigating DNA denaturation is well established[1–4]. This communication deals with the utilization of formaldehyde for characterizing the extent of strand-separation of calf-thymus DNA molecules heated up to different temperatures in the melting range and, more generally, for investigating the extent of complementary base-pairing in both partially denatured DNA molecules and mixtures of native and denatured DNA.

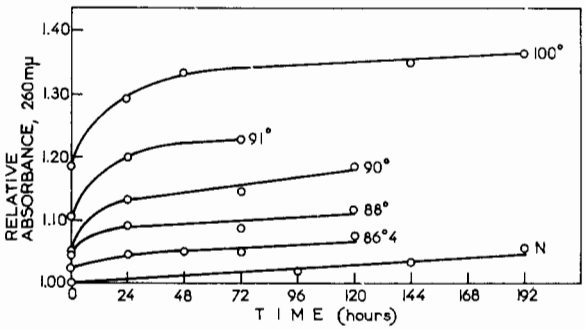


Fig. 1. Relative absorbance at 260 mμ of native DNA (N) and of DNA samples heated up for 15 min in 0.13 M NaCl–0.01 M potassium phosphate pH 6.8 to the different temperatures indicated in the figure, rapidly cooled and reacted with 1 % formaldehyde at 25°. Samples heated up to temperature comprised between 80° and 85° showed the same behavior of native DNA (see Fig. 2). Calf-thymus DNA was used in this experiment.

Calf-thymus DNA samples kept for 15 min at various temperatures ranging from 80° to 100° and rapidly cooled were reacted with 1 % formaldehyde at 25°, the solvent being 0.13 M NaCl–0.01 M potassium phosphate pH 6.8. Control DNA (indicated by N in Fig. 1) and DNA samples kept at temperatures comprised between 80° and 85° did not show any rapid hyperchromic shift upon addition of formaldehyde and only exhibited an extremely slow absorption increase over a period of several days. In contrast, samples kept at temperatures ranging from 86.4° to 100° showed an immediate increase in absorption which was higher in the samples heated up to higher temperatures. As shown in Fig. 1, this rapid increase was followed by a slower one over a period of 24–48 h when the formaldehyde reaction was carried out at 25°, whereas at 42° the reaction was complete in 2 h.

A plot of the absorption increases caused by the reaction with formaldehyde of DNA samples heated up to different temperatures against these temperatures has the shape of a DNA melting curve (Fig. 2). Two important points concerning this curve deserve to be commented upon.

The first one is that the hyperchromicity obtained by reacting DNA, heated up to 100° and fast-cooled, with formaldehyde is only equal to 34 %, whereas native DNA, when heated up to 100° in the presence of formaldehyde shows a hyperchromicity of 38–41 %. This difference is, in all likeliness, due to the fact that calf-thymus satellite DNA's rapidly undergo reassociation[5], like other satellite DNA's[6,7], when total DNA is fast-cooled in the absence of formaldehyde, whereas this reassociation

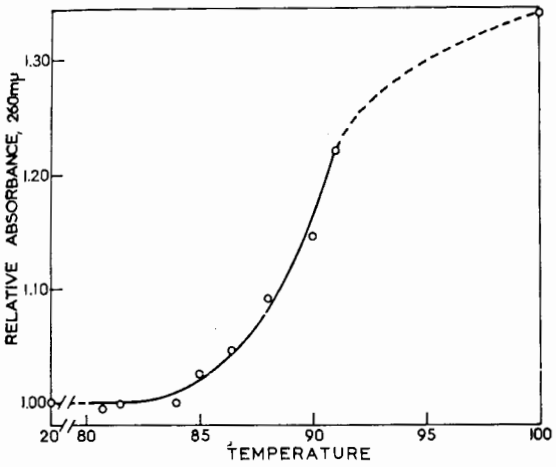


Fig. 2. Plot of the plateau values of $A_{260\ m\mu}$ obtained by reacting DNA samples heated up to different temperatures (see Fig. 1) with 1 % formaldehyde at 25° against these temperatures.

is prevented when heating and cooling take place in the presence of formaldehyde. In agreement with this explanation is the finding that when calf-thymus DNA is heated up to 100°, fast-cooled, reacted with formaldehyde and then heated again in the presence of formaldehyde it shows, as expected, a further hyperchromic shift of about 5 %, due to the reaction with formaldehyde of the satellite DNA whose strands had reassociated, forming regions of complementary base-pairing, during the fast-cooling in the absence of formaldehyde. An important conclusion to be drawn from these findings is that the curve of Fig. 2 only concerns the main DNA component of calf thymus.

The second point is that the transition shown in Fig. 2 has a mid-point close to 90°, a value definitely higher than the melting temperature, 87°, of calf-thymus DNA[8]. The difference is quite significant, specially considering that satellite DNA's from calf thymus, which have higher melting temperatures than the main DNA[5], do not contribute to the curve of Fig. 2, whereas they do so in the usual melting curve. The main satellite has a melting temperature of 93°; the minor satellite should have an even higher melting temperature since its buoyant density in CsCl density gradient is higher[5]. An explanation which may be suggested for this difference is that the curve of Fig. 2 is not a melting curve, but a strand-separation curve. In fact, only DNA molecules which have undergone a complete strand separation at the temperature at which DNA was heated, do not reform complementary base-paired structures upon cooling and, therefore, can react with formaldehyde. The other molecules, which only underwent a partial strand separation at the temperatures used in the step, certainly renatured during cooling since they still had paired regions.

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