Residual Transforming Activity
of Denatured *Haemophilus influenzae* DNA

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Appendix

An Electron Microscope Investigation on the Chromatographic
Fractions of Denatured Transforming DNA of *Haemophilus influenzae*

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When transforming *Haemophilus influenzae* DNA is denatured, a small percentage of its biological activity survives denaturation. It can be shown, by using hydroxyapatite chromatography, that the residual transforming activity is carried by about 10% of DNA molecules, which exhibit the chromatographic properties of native DNA and, therefore, can be separated from the bulk of inactive, denatured molecules.

The "native-like" fraction of DNA was investigated for its melting behavior, reversibility, renaturability, concentration–response curves and competitive ability against native DNA. In all these properties, this fraction is similar, yet not identical, with native DNA. It has been shown that the different properties of native-like DNA, compared to native DNA, cannot be solely explained by the fact that native-like DNA is contaminated by denatured material. The native-like molecules are, therefore, intrinsically different from native molecules; they appear to have disordered regions and/or single-stranded ends in otherwise double-stranded structures; furthermore, they are heterogeneous in their secondary structures. The native-like fraction seems to be formed by molecules the strands of which never came apart during the melting process, perhaps because of the existence of inter-strand cross-links of unknown nature.

1. Introduction

It is well known that when transforming DNA's from different bacteria are denatured, a small percentage of the original biological activity survives denaturation (Lerman & Tolmach, 1960; Marmur & Lane, 1960; Ginoza & Zimm, 1961; Roger & Hotchkiss, 1961; Herriott, 1961*a*,*b*). Understanding the structure and the origin of the DNA molecules carrying the residual transforming activity has proved to be a very difficult task. This is clearly indicated by the variety of mutually exclusive interpretations which have been put forward so far.

The residual activity was first thought to be the result of the specific re-association of a small number of separated, complementary, single-stranded DNA molecules, taking place during the rapid cooling of heat-denatured DNA solutions (Marmur & Lane, 1960), a view which led to the discovery of DNA renaturation; this interpretation was shown to be untenable, however, by the finding that the level of the residual activity is independent of the DNA concentration (Ginoza & Zimm, 1961), and of the ionic strength at denaturation (Roger & Hotchkiss, 1961). Subsequently, the residual activity was supposed to be carried by single-stranded DNA molecules,

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† This paper is the fourth in a series on *Chromatography of nucleic acids on hydroxyapatite.* The first three papers are by Bernardi and have been submitted for publication.

on the basis of results obtained in CsCl density-gradient centrifugations (Guild, 1961; Rownd, Lanyi & Doty, 1961); this view, too, was later shown to be incorrect, the residual activity being in fact carried by a small percentage of DNA molecules having properties similar to those of native DNA (Chevallier & Bernardi, 1965; Alberts, 1965).

Two more possibilities have been considered. It has been suggested that the residual activity is carried by a fraction of single-stranded DNA molecules folded upon themselves and possessing some extent of intra-strand hydrogen-bonding (Barnhart & Herriott, 1962; Barnhart, 1965). Alternatively, the activity might be due to a small fraction of DNA molecules which did not undergo strand-separation upon denaturation; these molecules have been thought to have a collapsed structure, or a structure very similar to that of native DNA (Roger & Hotchkiss, 1961; Rownd, Green & Doty, 1963, *Abstr. Biophys. Soc. TB7*; Marmur, Rownd & Schildkraut, 1963; Chevallier & Bernardi, 1965; Alberts, 1965).

We thought that hydroxyapatite chromatography, a technique which fractionates polynucleotides according to their secondary structure (Bernardi, 1961,1962,1965; and manuscripts submitted for publication), was ideally suited to investigate the problem of residual transforming activity. In fact, HA† chromatography immediately showed that the residual activity was carried by a small fraction of DNA molecules which exhibited the chromatographic properties of native DNA (Chevallier & Bernardi, 1965). This finding was very interesting in two respects: (a) it disposed of the hypothesis that activity was carried by single-stranded DNA molecules having a decreased efficiency of incorporation; (b) it showed that the activity was carried by a native-like fraction already shown to be present in DNA's from higher organisms and from bacteria (Bernardi, 1962,1965, and manuscript submitted for publication), thus indicating that the problem under investigation was, in fact, not restricted to transforming DNA.

We report here an investigation on the native-like DNA fraction carrying the residual transforming activity, as isolated by HA chromatography.

2. Materials and Methods

(a) *Cells and transformation*

Haemophilus influenzae (Rd) cells, sensitive to antibiotics, were used as the recipient strain. Cells resistant to 1 μg of cathomycin/ml. were used as the donor strain in most experiments. Alternatively, cells resistant to 1 μg of cathomycin/ml., 8 μg of erythromycin/ml., 200 μg of viomycin/ml. and 1 mg of streptomycin/ml. were used.

The transformation assay has been already described elsewhere (Bach, Luzzati & Chevallier, 1966). Competent cells were prepared according to Barnhart & Herriott (1963). Transformation experiments were done at non-saturating DNA levels (0·01 or 0·05 μg/ml.), except where otherwise stated.

(b) *DNA*

DNA was prepared according to Bach *et al.* (1966). Several DNA samples were used in the present work; some of the properties of the four samples most extensively used are given in Table 1.

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† Abbreviations used: HA, hydroxyapatite; KP, potassium phosphate buffer (pH 6·8); SSP, standard saline phosphate, is 0·15 M-NaCl–0·01 M-sodium phosphate buffer (pH 7·0).

In this paper we call fraction 1 and fraction 2 the two chromatographic fractions of denatured DNA obtained from HA columns; the native-like fraction is the DNA component present in fraction 2, showing properties similar to native DNA.

TABLE 1

Properties of the DNA samples

	$M_w \times 10^{-6}$†	s‡	Protein § (%)	Genetic markers
N_1	6·5	21·0	0·74 ⎱	Cathomycin
N_2	10·1	26·0	0·83 ⎰	
$EVSN_G$	—	21·7	1·25 ⎱	Erythromycin, viomycin,
$EVSN_{332}$	6·8	21·2	0·4 ⎰	streptomycin, cathomycin

† Light-scattering molecular weight. The molecular weight of sample N_2 is probably under-estimated (Froelich, Strazielle, Bernardi & Benoit, 1963).

‡ Sedimentation coefficient, as determined in a Spinco analytical ultracentrifuge equipped with ultraviolet optics.

§ Using a modified Lowry method (Ramachandran & Fraenkel-Conrat, 1958).

(c) *Other methods*

Formaldehyde treatment, melting curves, physical measurements, and HA chromatography and denaturation of DNA were carried out as described elsewhere (Bernardi, manuscript submitted for publication). Heat-denaturation was done by immersing Erlenmeyer flasks containing the DNA solutions (10 to 40 μg/ml. in 0·13 M-NaCl–0·01 M-potassium phosphate, pH 6·8) in a boiling water bath for 10 min; solutions were then rapidly cooled by pouring them into flasks kept in an ice bath. Alkaline denaturation of DNA was performed at room temperature by titrating DNA solutions, at the concentrations indicated above, to pH 12·5. Neutralization was done with 0·5 M-KH_2PO_4 or sodium phosphate buffer, pH 6·8.

3. Results

(a) *Residual transforming activity of denatured DNA*

A first series of experiments was aimed at verifying the effect of several different conditions used in heat-denaturation on the residual transforming activity. The effects of DNA concentration and of denaturing temperatures were studied; the results obtained are given in Table 2. They show that the residual transforming activity is independent of DNA concentration, for concentrations lower than, or equal to, 30 μg/ml., and is still detectable after heating at 110 and 120°C.

The time-course of heat inactivation of transforming activity (Fig. 1) is characterized by a very sharp drop in the first few minutes, followed by a much slower decrease. This latter phenomenon is due to depurination and hydrolysis of DNA, whereas the initial drop is caused by the melting of the secondary structure. By extrapolating the slow inactivation curve back to zero time, one finds that the initial transforming activity is decreased to about 4% by heat denaturation. Similar results have been obtained by studying the time-course of alkali denaturation (Fig. 7, curve B); in this case, the residual activity of alkali-denatured DNA has been found equal to, or very slightly higher than, that of heat-denatured DNA after extrapolation to zero time of treatment (compare Fig. 1 and Fig. 7, curve B).

Residual transforming activity is independent of the ionic strength in the range of 0·005 to 0·15 M-NaCl, provided that values extrapolated to zero heating time are used. The rates of inactivation caused by depurination and hydrolysis increase as the ionic strength is lowered. The ratio of these rates, as determined at 0·01 and 0·15 M-NaCl,

TABLE 2

Effects of DNA concentration and denaturing temperature on the residual transforming activity

DNA concentration (μg/ml.)	Activity (%)
0·01	1·50
0·10	1·45
1·0	1·42
10	1·50
20	1·60
30	1·20

Denaturing temperature (°C)	
100	1·1
110	1·0
120	0·34

Transformation was carried out at a single DNA concentration (0·01 μg/ml.). Activity values are given as percentage of the activity of starting, native DNA; no correction for heat degradation was applied.
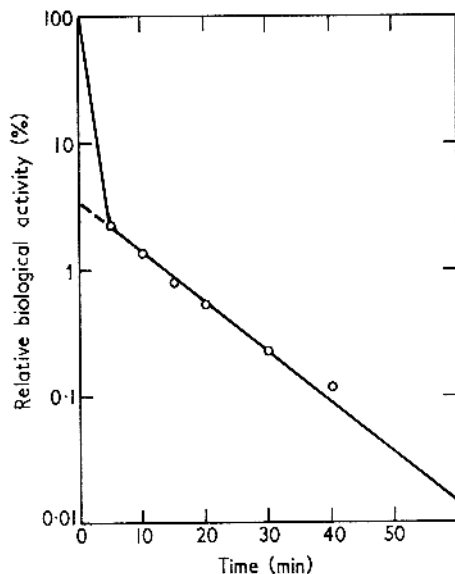
FIG. 1. Inactivation of cathomycin marker at 100°C.

A sample of 3 ml. of DNA (sample N_1; 18 μg/ml. in 0·15 M-NaCl–0·01 M-sodium phosphate buffer, pH 7·0, SSP), was immersed in a boiling water bath. At the times indicated, 0·1-ml. portions were withdrawn and immediately diluted with 35 vol. of cooled solvent. Biological activity was tested at a DNA concentration of 0·05 μg/ml.

was found to be 2; interestingly enough, this is also the ratio of the depurination rates at these two ionic strengths, as calculated from the data of Greer & Zamenhof (1962).

No significant differences have been found in the residual activities shown by three different genetic markers. These were shown to melt at temperatures very close to 88°C (Fig. 2).
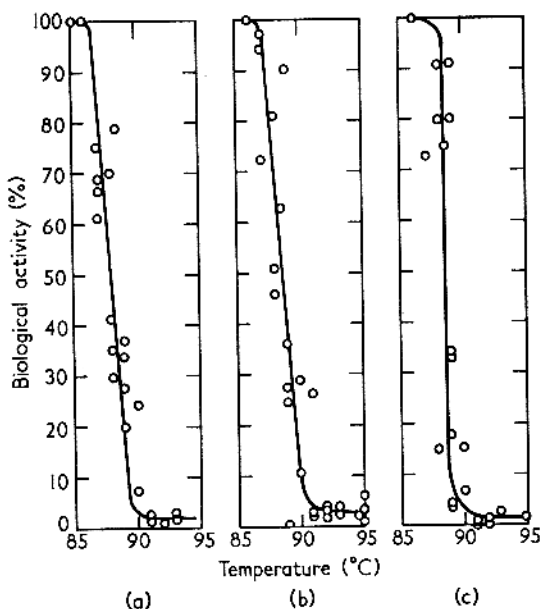

Fig. 2. Thermal inactivation of three *H. influenzae* genetic markers: streptomycin (a), cathomycin (b) and erythromycin (c).

2 ml. of DNA sample EVSN₁ (20 μg/ml. in SSP) were kept for 15 min at the temperatures indicated and then quickly cooled. Biological activity was tested at a DNA concentration of 1 μg/ml. Melting temperatures were found to be 88°C for the streptomycin marker, 88·6°C for the cathomycin and the erythromycin markers.

(b) *Fractionation of denatured DNA on hydroxyapatite columns*

Heat or alkali-denatured *H. influenzae* DNA is eluted from HA columns by a gradient of potassium phosphate buffer (pH 6·8) in two fractions: a large one which is eluted at about 0·12 to 0·15 M-KP (fraction 1) and a small one, which is eluted at 0·21 to 0·23 M-KP as a peak or as a shoulder of fraction 1 (fraction 2). The recoveries of optical density and biological activity from the columns have been, in most cases, 60 to 90%. When the residual activity of a DNA sample carrying four genetic markers was investigated by HA chromatography, it was found that all the residual activities were present in fraction 2 and that no fractionation of these markers occurred within the peak.

Figure 3(b) shows this chromatographic behavior, which is different from that shown by native *H. influenzae* DNA, the latter being eluted in a single peak, centered at about 0·22 M-KP (Fig. 3(a)). The elution patterns shown by both native and denatured *H. influenzae* DNA are those typically exhibited by native and denatured DNA samples, respectively, from bacteria and higher organisms (Bernardi, 1965 and manuscripts submitted for publication).
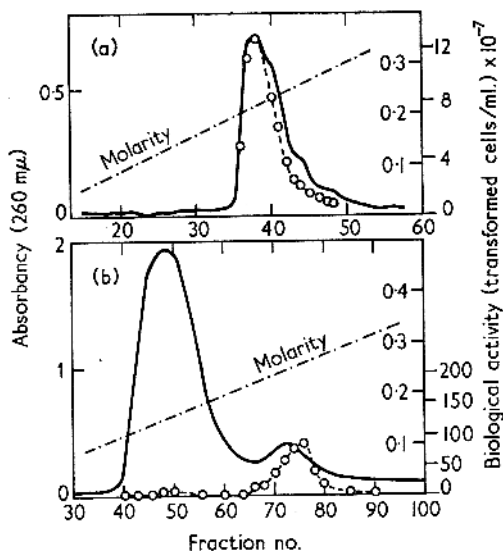
Fig. 3. (a) Chromatography of native DNA: 1 ml. of sample $EVSN_1$ in SSP, $A_{260} = 16.7$, was loaded on a 1 cm $\times$ 10 cm HA column. Elution was carried out with a linear gradient (100 ml. + 100 ml.) of KP, molarity (0·001 to 0·5 M, inner scale); 3-ml. fractions were collected. Recovery of optical density was 85%; recovery of biological activity (cathomycin marker; circles; right-hand outer scale) 82%.

(b) Chromatography of alkali-denatured DNA: 1850 ml. of sample N_2, $A_{260} = 0.048$, were loaded on a 1·2 cm $\times$ 20 cm HA column. Elution was carried out with a linear gradient (150 ml. + 150 ml.) of KP, molarity (0·001 to 0·5 M, inner scale);2·4-ml. fractions were collected. Recovery of optical density was 103%; recovery of biological activity (cathomycin marker; circles; right-hand outer scale) 98%. The specific activity relative to untreated DNA was 43% for fraction 76, which exhibited the highest relative activity, and 29% for fractions 66 to 85 (average value); these values were corrected for contamination by fraction 1.

Practically all the residual transforming activity is associated with fraction 2, fraction 1 being essentially inactive (Fig. 3(b)). The elution curves suggest that fraction 2 is contaminated by the tailing of the much larger fraction 1. This contamination is clearly demonstrated by the following experiments. (a) Rechromatography of fraction 2 shows that the native-like material of this fraction is contaminated by important amounts of denatured DNA from fraction 1. This extent of contamination could be estimated as comprising, in general, between 30 and 50%; the contamination could be even larger in fractions 2, eluting as shoulders of the main peak of denatured DNA (Fig. 4(a)). (b) A comparison of the activity and optical density profiles obtained in the chromatograms shows that they do not coincide, the biological activity peak always following the optical density peak (Fig. 3(b)). Interestingly enough, this occurred even in the rechromatography experiments (Fig. 4(b)).

After correction for contaminating material from fraction 1 and difference in ultraviolet absorption, the amount of native-like DNA was estimated to form about 10% of total DNA. This estimate was based on three experiments in which recoveries of both biological activity and optical density were higher than 90%. The average activity, determined at a non-saturating DNA concentration, and corrected for heat damage (in the case of heat-denatured samples), was about 40% relative to that of
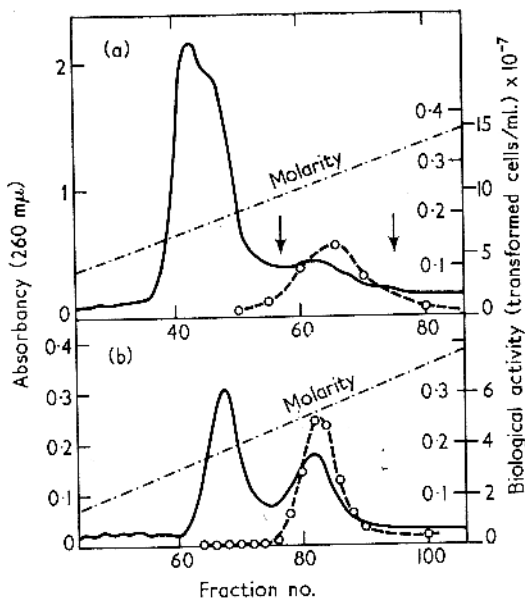
FIG. 4. (a) Chromatography of alkali-denatured DNA: 200 ml. of a DNA solution (sample N_2A; 37 μg/ml. in SSP) were adjusted to pH 12·8 with 5 N-NaOH at room temperature. After about 10 min at this pH, the solution was neutralized with 2 M-KH_2PO_4, diluted with SSP to 200 ml. and loaded on a 1 cm × 20 cm HA column. Elution was carried out with a linear molarity gradient (150 ml. + 150 ml.) of KP (0·001 to 0·5 M, inner scale); 2·4-ml. fractions were collected. Recovery of both optical density and biological activity (cathomycin marker, shown as circles) was 51%.

(b) Rechromatography of fraction 2 from previous chromatography; fractions 57 to 75 (between arrows) were pooled, adjusted to 400 ml. with SSP and loaded on a 1 cm × 10 cm HA column. Elution was carried out with a linear molarity gradient (100 ml. + 100 ml., inner scale) of KP; 2·4-ml. fractions were collected. Recovery of optical density was 60%, of biological activity 49%.

native DNA. This value is that expected on the basis of total residual activity (4%) and amount of native-like fraction (10%).

These results indicate that the DNA molecules carrying the residual activity are eluted at the same molarity as native DNA. This point was further checked by running an artificial mixture of native DNA obtained from streptomycin-resistant cells and denatured DNA from cathomycin-resistant cells. The results, shown in Fig. 5, clearly indicate that no separation of native DNA and the native-like fraction of denatured DNA could be obtained, at least with the technique used in the present work, which may be considered to be a low-resolution technique (Bernardi, manuscripts submitted for publication). It is interesting to notice that the transforming activity of renatured DNA is also eluted at the same molarity as native or native-like DNA.

(c) *Properties of the chromatographic fractions of denatured* H. influenzae *DNA*

Several properties of the two DNA fractions obtained from HA columns were investigated. Some difficulties were experienced with fraction 2 because of its low concentration and the small amounts available. The following results were obtained.

Melting curves of the two chromatographic fractions are shown in Fig. 6(b). Figure 6(a) shows, for comparison, the melting curves obtained with native (curve A), unfractionated, heat-denatured (curve B) and unfractionated renatured *H. influenzae* DNA (curve C). The melting curves shown by fraction 2 exhibit a sharp melting
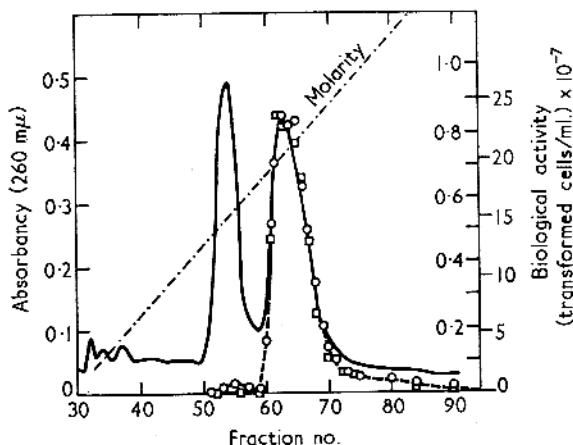

FIG. 5. Chromatography of a mixture of native (streptomycin marker) and heat-denatured DNA (cathomycin marker).

31 ml. of a DNA solution (sample N_2; 25 μg/ml. in SSP) were heat-denatured, added to 5 ml. of a native DNA solution (sample S; 75 μg/ml. in SSP), and loaded on a 2 cm $\times$ 15 cm HA column. Elution was carried out with a linear molarity gradient (100 ml. + 100 ml.) of KP (0·001 to 0·5 M); 2·7-ml. fractions were collected. Circles indicate the cathomycin activity (right-hand, inner scale); squares indicate the streptomycin activity (right-hand, outer scale). The elution molarity of the first peak was 0·14 M; that of the second peak, 0·21 M. Recovery of optical density was 76%; recovery of streptomycin activity, 76%; recovery of cathomycin activity, 62%. Biological activity was tested at a DNA concentration of 0·05 μg/ml.
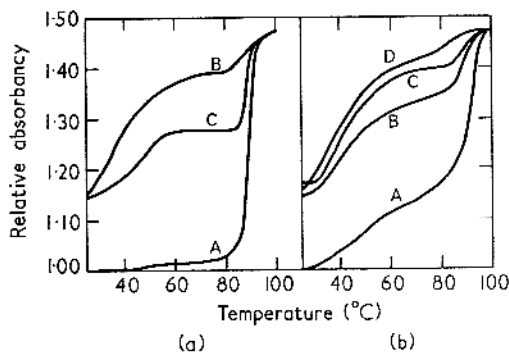

FIG. 6. (a) Ultraviolet melting curves of *H. influenzae* DNA in SSP: curve A, native DNA (sample N_1); curve B, heat-denatured DNA (sample N_2); activity was 2·2% of native; curve C, renatured DNA (sample N_1); activity was 34% of native.

(b) Ultraviolet melting curves of chromatographic fractions of *H. influenzae* heat-denatured DNA in 0·1 to 0·2 M-KP. Curve A, fraction 2; this sample was from the A_{260} peak tube (DNA sample $EVSN_G$) as obtained from a well-separated fraction; activity was 20% of native. Curve B, fraction 2; this sample was from the A_{260} peak tube (DNA sample N_2) as obtained from a poorly separated fraction; activity was 15% of native. Curve C, fraction 1; this sample was from an early tube of the fraction (DNA sample N_2); activity was 0·12% of native. Curve D, fraction 1; this sample was the peak tube of the fraction (DNA sample N_2); activity was 0·14% of native. Curves B, C and D were obtained on fractions derived from the same chromatography.

region between 80 and 100°C and a continuous increase in the 40 to 80°C temperature range (Fig. 6(b), curves A and B). The results obtained with fraction 2 samples originating from different chromatographic experiments showed rather conspicuous differences: two different cases, curve A, in which the separation of the two fractions was excellent, and curve B, in which it was less clear-cut, are shown in Fig. 6(b). It is evident that samples obtained from well-separated native-like components show sharper melting profiles and larger hyperchromic shifts than samples obtained from chromatograms in which the separation was poor.

In contrast, fraction 1 exhibited a melting curve typical of denatured DNA with a continuous increase in optical density across the whole temperature range, and a small hyperchromic shift (Fig. 6(b), curves C and D). Small, yet noticeable, differences were found in the melting curves of early, mid- and late fractions in the denatured DNA peak: a small, relatively sharp-melting, region could be seen in mid- or late fractions (Fig. 6(b), curve D), but not in early ones (Fig. 6(b), curve C). These results are similar to those obtained with calf thymus DNA by Bernardi (manuscript submitted for publication).

Alkali denaturation of fraction 2 from heat-denatured DNA caused a drop in activity to about 30% of the original value; the same treatment on native DNA provoked a decrease in activity to about 4%, as already mentioned (Fig. 7).
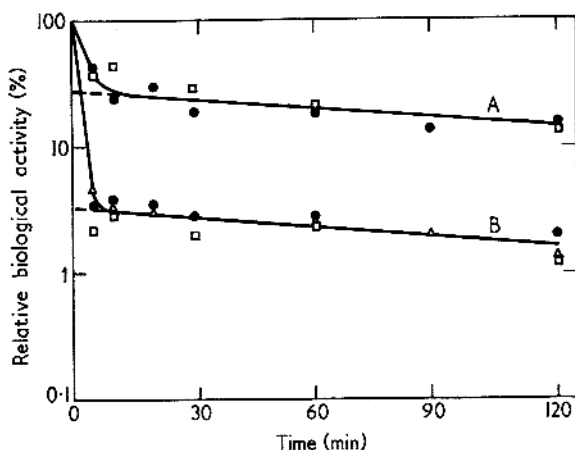

FIG. 7.   Alkali-denaturation of fraction 2 of heat-denatured DNA (curve A);   native DNA (curve B). In both cases DNA (sample N_1; cathomycin marker; 1 μg/ml. in SSP) was adjusted to pH 12·5 by addition of 0·1 N-NaOH. At the times indicated, 0·1-ml. portions were neutralized with 10 vol. of 0·1 M-sodium phosphate buffer (pH 7). Biological activity was tested at DNA concentration of 0·01 μg/ml. Different signs refer to different experiments.

These results indicate that fraction 2 from heat-denatured DNA withstands alkali denaturation much better than native DNA; yet its "reversibility" (Geiduschek, 1961) is far from complete, probably because of strand breakage in alkaline medium at the level of depurinated sites.

Renaturation experiments were carried out, at two different DNA concentrations, on unfractionated, denatured DNA, and on the two chromatographic fractions. The results obtained (Fig. 8) indicated that, in contrast to unfractionated DNA and to fraction 1, which showed linear activity increases with time and with DNA con-
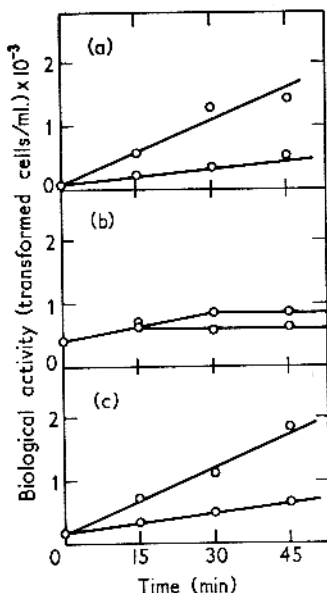
Fig. 8. Renaturation time-course of heat-denatured DNA (sample N_2; cathomycin marker). (a) Fraction 1; (b) fraction 2; (c) unfractionated. In all cases, the upper curves refer to a DNA concentration of 10 μg/ml., the lower ones to 2 μg/ml. All samples were dialyzed against 0·3 M-NaCl–0·03 M-sodium citrate and immersed in a water bath at 66°C; portions were withdrawn at the times indicated and diluted with cold SSP so as to obtain a final DNA concentration of 0·01 μg/ml. for the transformation assays.

centration, fraction 2 only showed a small increase in activity with time and much less dependence on DNA concentration.

Competition experiments were done by measuring the capacity of (a) native DNA, (b) ultraviolet-irradiated DNA and (c) fractions 1 and 2 of heat-denatured DNA (all carrying the cathomycin-resistance marker), to decrease the transforming activity of native DNA, carrying the erythromycin-resistance marker. Results are displayed in Fig. 9 and show that fraction 1 is practically non-competitive, whereas fraction 2 exhibits a very important degree of competitiveness, albeit not as high as native or ultraviolet-irradiated DNA.

Concentration–response experiments were performed with the two chromatographic fractions and with native DNA. The results are shown in Fig. 10. Fraction 1 approached saturation at a DNA concentration more than 100 times higher than that of native DNA. It is impossible to estimate the plateau activity of fraction 1, since saturation was not attained even with the highest DNA concentration used. In contrast, fraction 2 reached a plateau value close to 75% of that of native DNA, at a concentration about five times higher; these data were calculated after due correction for DNA inactivation by depurination and hydrolysis; without correction, the plateau value was 28%. In this experiment, fraction 2 material originated from two fractions located at the peak of optical density.

An important point shown by this experiment was that the biological activity, relative to that of native DNA, shown by fraction 2 at a non-saturating concentration was (after correction for the presence of contaminating denatured DNA) about three times lower than at saturation.
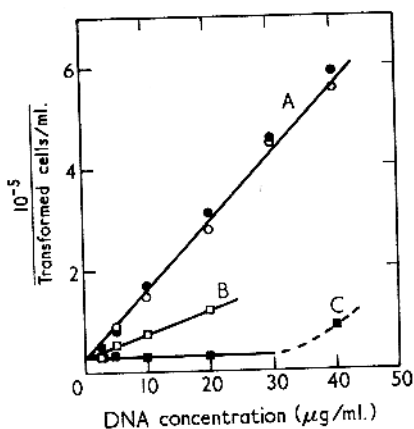
FIG. 9. Competitive ability: curve A, native DNA (filled circles) and ultraviolet-irradiated DNA (3000 ergs/mm²; 2537 Å; surviving transforming activity: 4·6%; open circles). Curve B, heat-denatured DNA, fraction 2 (open squares). Curve C, heat-denatured DNA, fraction 1 (filled squares). Increasing concentrations, indicated on abscissa, of competitive DNA (sample N_1; cathomycin marker) were added to native DNA (sample E_1; erythromycin marker; saturating concentration of 1 μg/ml.).

The value obtained at 40 μg/ml. of denatured DNA is suspect, due to a decreased competence of the cells caused by the fact that undiluted DNA, saturated with chloroform–isoamyl alcohol, was used in this experiment.
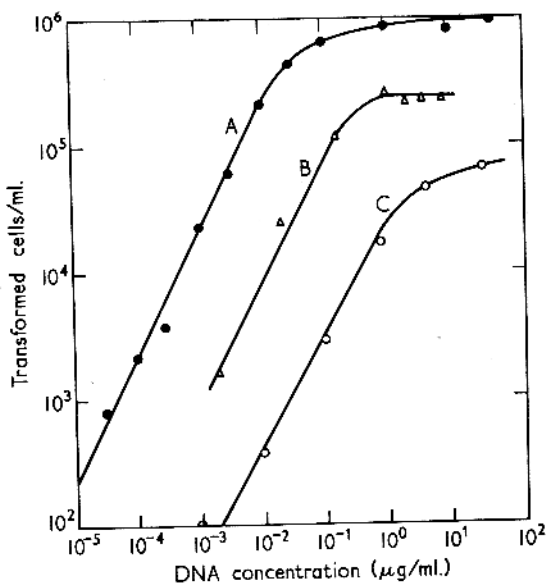

FIG. 10. Transformation at different DNA concentrations (sample N_1; cathomycin marker). Curve A, native DNA; curve B, heat-denatured DNA, fraction 2; curve C, heat-denatured DNA, fraction 1.

The fraction 2 material used in this experiment was derived from two fractions located at the peak of optical density. Contamination by denatured DNA was estimated at 30 to 40%.

Electron microscope experiments on the two chromatographic fractions are reported in the Appendix.

4. Discussion

(a) *Residual transforming activity of denatured DNA*

No special comments are needed on our results on the non-dependence of the residual transforming activity upon DNA concentration and ionic strength of DNA solvent and those on the temperature of denaturation of DNA, since they confirm results already obtained by previous authors (Ginoza & Zimm, 1961; Roger & Hotchkiss, 1961; Marmur & Lane, 1960).

The time-course of heat-inactivation shows a typical biphasic pattern indicating that two inactivating processes are operative at the same time: DNA melting and DNA depurination and hydrolysis. Similar results have also been obtained by Goodgal (1961), Becker, Zimmerman & Geiduschek (1964) and Alberts (1965).

(b) *Fractionation of denatured DNA on hydroxyapatite columns*

The chromatographic experiments on denatured transforming DNA have shown that this material exhibits the usual behavior of all denatured DNA's from bacteria and higher organisms tested so far (Bernardi, 1962,1965, and manuscripts submitted for publication), since it is separated into a main fraction eluting at a molarity of 0·12 to 0·15 m-KP and a minor fraction eluting, like native DNA, at a molarity of 0·21 to 0·23 m-KP. Experiments in which native DNA was co-chromatographed with denatured or renatured DNA showed that no separation occurred between native DNA and the native-like fraction or renatured material.

(c) *Properties of the chromatographic fractions of denatured DNA*

In general, the properties of fraction 1 are closely similar to those of unfractionated, denatured DNA; this is scarcely surprising, since fraction 1 forms about 90% of denatured DNA. Obviously, a basic difference between fraction 1 and total denatured DNA is that fraction 1 is practically devoid of transforming activity.

In contrast, the properties of fraction 2 are similar to, yet not identical with, those of native DNA. In principle, contamination of the native-like fraction by denatured DNA (shown by both chromatography and electron microscopy) might explain the different properties exhibited by fraction 2, when compared to native DNA. The competition results and the concentration–response curves, however, clearly indicate that contamination by denatured DNA cannot be the only explanation for these differences. In fact, contamination has been estimated (on the basis of chromatographic data) to comprise between 30 and 40% in the fraction 2 samples used in the experiments reported. These values are very significantly lower than those which can be calculated assuming that fraction 2 is a mixture of fully native and fully denatured molecules. For this explanation to be valid, contamination should have been as high as 65% in the competition test, and close to 80% in the concentration–response experiment.

Having ruled out contamination by denatured DNA as the sole explanation for the differences between fraction 2 and native DNA, we are led to conclude that native-like molecules are intrinsically different from native molecules. We may add: (a) that they have a double-stranded structure characterized by disordered regions

and/or single-stranded ends; (b) that they are heterogeneous in their secondary structures. The following lines of evidence may be quoted in support of these conclusions.

(a) The chromatographic behavior of the native-like molecule is very similar to that of native, renatured and cross-linked DNA, from which it cannot be distinguished, at least with the technique used in the present work. An indication as to the heterogeneity of its secondary structure is given by the finding that the specific transforming activity is not constant in fraction 2 but increases in the later eluting fractions (after correction for contamination by denatured DNA). This was found also in the re-chromatography experiments.

(b) Electron microscopy (see Appendix) shows the presence in fraction 2 of double-stranded molecules, which may end in single-stranded filaments; other possible imperfections in the secondary structures of the native-like molecules are not expected to be seen with the technique used.

(c) In the concentration–response experiment, fraction 2 reaches a plateau value close to 75% of that of native DNA (after correction for heat damage). This indicates that the activity level of the "best" DNA molecules present in the particular fractions which were examined (see Results), is significantly lower than that of native DNA; this result implies, in turn, that 25% of the molecules which enter the cells, at saturation, are unable to transform. This percentage becomes much more important when operating under non-saturating conditions, since the specific activity of fraction 2 (corrected for the presence of 30% contaminating denatured DNA) relative to that of native DNA was found to be lower by a factor of 3 when measured at non-saturation. These results are further indications of heterogeneity and show that some of the molecules may have the structural requirements for cell penetration and yet be unable to transform. In contrast, it is likely that fractions from the tail of the eluted material might reach the same saturation value as native DNA, since they showed much higher values than average at non-saturation.

A very important question concerns the origin of the native-like DNA fraction. The hypothesis put forward by Barnhart & Herriott (1962) and Barnhart (1965), that this fraction is formed by single-stranded molecules folded upon themselves and having some extent of intra-strand hydrogen bonding, is compatible with the results we have obtained on the fraction, with the possible exception of the electron microscopic data, which fail to show loops or other gross irregularities in the native-like molecules. A major difficulty of this model is, however, that the ability to form such structures is required to be uniformly distributed in all DNA molecules, since all genetic markers show the same residual activity.

An alternative explanation (Chevallier & Bernardi, 1965; Alberts, 1965) is that the native-like fraction is formed by DNA molecules with strands which never came apart during denaturation. In this case, it is essential to understand what holds the two strands of a small percentage of DNA molecules together. Certainly this is not G–C-rich regions, since the residual activity can be found after heating to temperatures which melt dG–dC polymers and after alkaline denaturation to which GC pairs are sensitive (Murray & Peacocke, 1962; Inman & Baldwin, 1964). On the basis of work done on the residual transforming fraction of *Bacillus subtilis* DNA, as isolated by the two-phase system of Albertsson (1962), Alberts (1965) has suggested that the DNA molecules responsible for the residual activity have inter-strand cross-links. It should be pointed out that in *B. subtilis* only fully native molecules have substantial activity

in transformation and that the fractionation system used by Alberts selects the "best" DNA molecules, which have essentially the same properties as native DNA.

Our conclusions on the secondary structure of the *H. influenzae* residual transforming fraction may seem to be incompatible with the cross-link hypothesis. They can be reconciled with it, however, if one considers the following points: (a) The heat-denaturation procedure, which we have mostly used in our work, introduces in the DNA molecules random breaks, which add to possibly pre-existing ones. This phenomenon may be responsible for the "loss" of large single-stranded fragments upon cooling and explain the finding of single-stranded ends in the native-like molecules. (b) In the *H. influenzae* system, partially denatured molecules are active in transformation, as shown by Mulder & Doty (C. Mulder, personal communication, 1967) and ourselves. (c) Our fractionation system is not very sensitive to the presence of disordered regions in otherwise double-stranded DNA molecules. (d) The chromatography of the native-like molecules is very similar to those exhibited by native, renatured and cross-linked DNA (Bernardi, manuscript submitted for publication).

In conclusion, cross-links of a yet unknown nature seem to be, at the present time, the least objectionable explanation for the origin of the DNA fraction responsible for the residual transforming activity. It is quite evident, however, that additional work is needed for a complete elucidation of the problem.

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APPENDIX

An Electron Microscope Investigation on the Chromatographic Fractions of Denatured Transforming DNA of *Haemophilus influenzae*

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In view of the results presented above, we have investigated, using the electron microscope, the structure of the two chromatographic fractions of denatured transforming *H. influenzae* DNA. Native and denatured DNA were investigated for the sake of comparison. Samples were prepared according to Kleinschmidt & Zahn (1959), Freifelder & Kleinschmidt (1965), Nicolaieff & Sadron (1967). In order to avoid possible aggregation, a low concentration of DNA was used (0·5 µg/ml.). DNA was visualized by evaporating about 30 mg of uranium on the rotating sample at an angle of 6 to 9° and a distance of 120 mm.

The results presented here are a fair representation of the DNA structures as observed in many samples; however, no attempt to get quantitative data was made.

Native DNA appears as long filaments (Plate I(a)); if spreading is done just after preparing the DNA–protein mixture, filaments have a flower-like appearance. DNA denatured in the presence of formaldehyde appears as filaments which are shorter than native ones; DNA denatured in the absence of formaldehyde appears as collapsed structures with only very few filaments present (Plate I(b)).

As expected, fraction 1 has the appearance of collapsed DNA (compare Plates III(c) and I(b)). This type of structure has been previously observed when using a different preparative technique (Doty, Marmur, Eigner & Schildkraut, 1960; MacLean & Hall, 1962; Bartl & Boublik, 1965).

In contrast, fraction 2 contains filaments (Plate II(a)), some of which are very long (Plate II(b)). When fraction 2 was obtained from poorly separated chromatographic fractions, collapsed structures were found together with filaments (Plate III(a)). It was observed that some filaments ended in collapsed structures (Plate III(b)). The continuity observed between filaments and collapsed structures suggests that single-stranded ends exist in some of the molecules. Since the resolution of the method used is only 150 to 200 Å, the possibility exists that these structures are due to denatured fragments lying next to native-like molecules. This seems to be ruled out, however, by the fact that single-stranded ends were not more frequent in unfractionated, denatured DNA, where the ratio of denatured to native-like molecules is much higher.

The filaments of fraction 2 are thought to be formed by double-stranded structures; in fact, since no formaldehyde was added, fraction 2 would show up like fraction 1 if it were formed by single-stranded DNA.

In conclusion, these results support the idea that the biologically active fraction of denatured transforming DNA has a double-stranded, native-like structure; some of the molecules have single-stranded ends.

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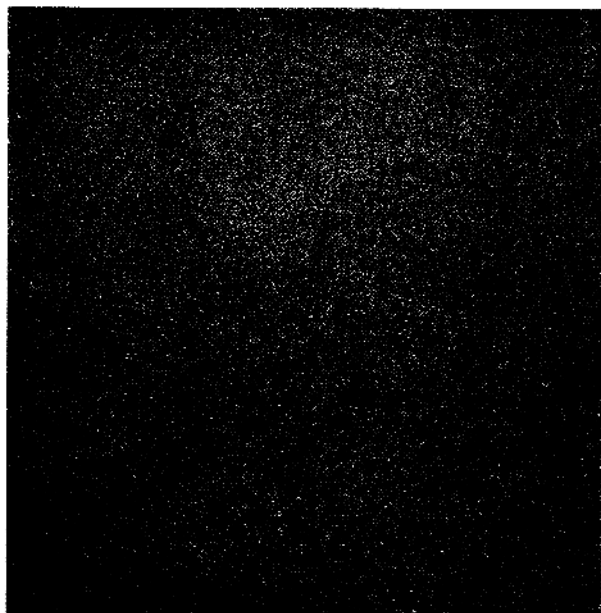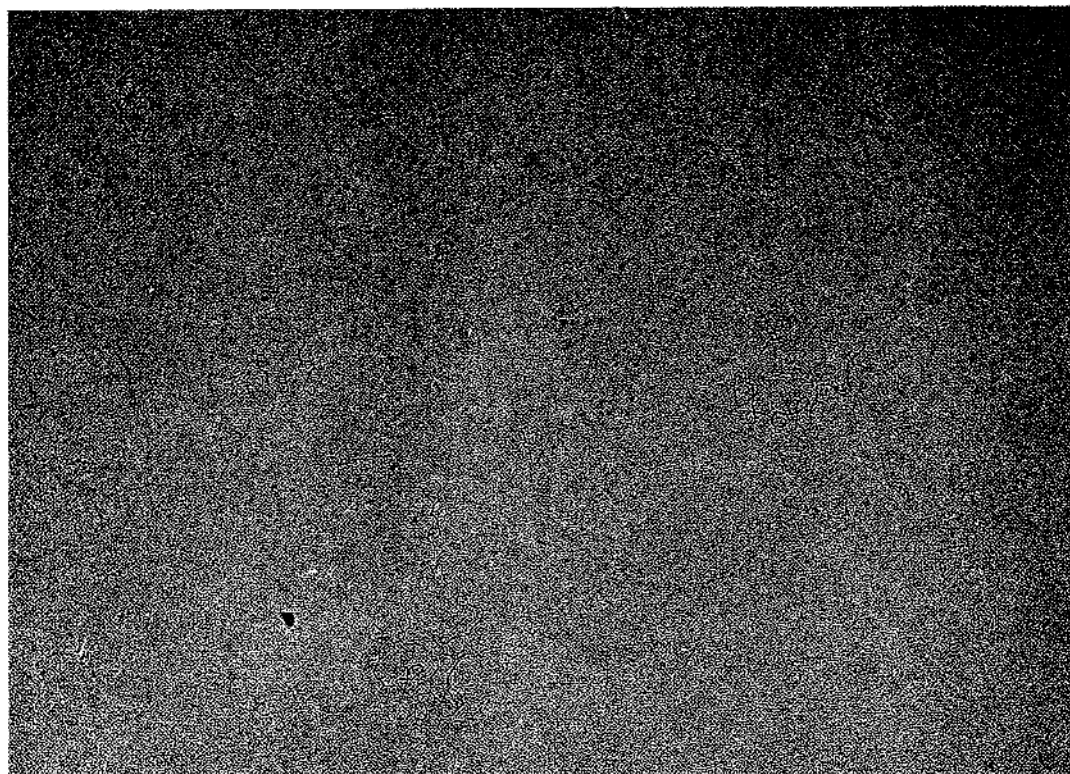
(a)

(b)

PLATE I. (a) Native *H. influenzae* DNA. The mixture of DNA and cytochrome *c* was kept 24 hr at room temperature before spreading. Note the extended structure. ×24,000.
(b) *H. influenzae* DNA, heat-denatured and quickly cooled. ×24,000.

(a)

(b)

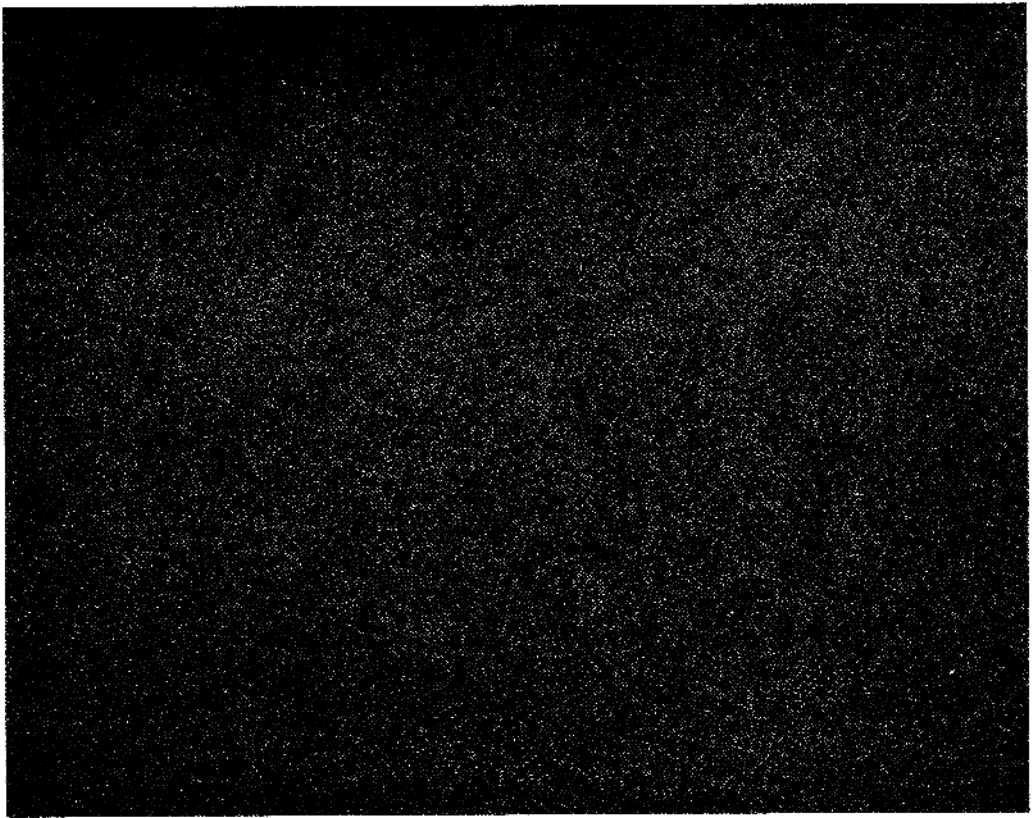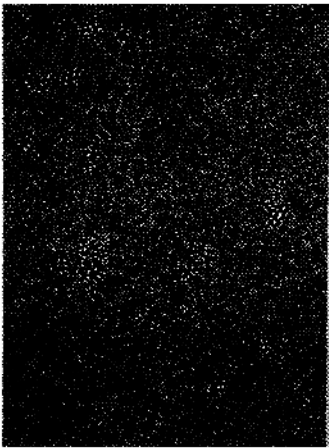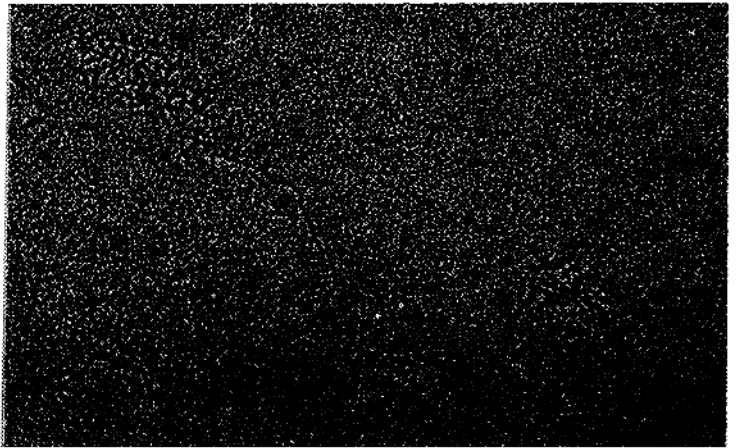PLATE II. (a) *H. influenzae* DNA. Heat-denatured, fraction 2. ×24,000.
(b) Fraction 2, a particularly long filament (5·5 μ). ×24,000.

PLATE III. (a) *H. influenzae* DNA. Alkali-denatured, fraction 2. The chromatographic separation of the two fractions was not as good as for the sample of Plate II(a).

(b) Fraction 2, enlarged detail of the same sample as in III(a). Note the continuity between filament and collapsed structure.

(c) Fraction 1. Alkali-denatured DNA. Only collapsed structures are present.