Resolution of Partially Denatured Deoxyribonucleate into Native and Denatured Components

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(Received for publication, October 17, 1963)

The development of procedures for fractionation of tissue deoxyribonucleate has received considerable attention since the demonstration (1, 2) of the compositional heterogeneity of such DNA. Earlier findings (3, 4) that the electrophoretic mobilities of native and denatured DNA were somewhat different suggested that zone electrophoresis might be used for separation of native and denatured DNA, and that, combined with procedures for partially denaturing DNA, it might provide another method of achieving a degree of compositional fractionation. A further purpose of the present work was to gain additional information on the extent to which partially denatured DNA consists of fully native, fully denatured, or partially denatured molecules.

EXPERIMENTAL PROCEDURE

Calf thymus DNA used in these studies was made by previously described procedures (5, 6). Thermal denaturation was carried out by heating solutions (usually containing about 1 mg of DNA per ml in 0.001 M NaCl-10-4 M EDTA; pH 7) for 10 minutes at 100° or at appropriate lower temperatures for partial denaturation; the solutions were cooled by immersing the test tubes in an ice bath. For acid or alkaline denaturation, the pH was adjusted to the desired value by careful addition of either dilute HCl or NaOH and the solutions were then rapidly neutralized by addition of the calculated amount of either K2HPO4 or a mixture of KH2PO4 and HCl. The extent of denaturation was estimated as described elsewhere (7).

Electrophoretic separations were carried out in an LKB model 3340 column electrophoresis apparatus as described by Svensson (8). The stabilizing gradient was 0 to 52% sucrose. The sample (about 2 mg of DNA in 2 ml of dilute salt solution) was introduced automatically as part of the least dense portion of the sample (about 2 mg of DNA in 2 ml of dilute salt solution) was introduced automatically as part of the least dense portion of the sample. A total of 300 ml was introduced. The acid and alkali locks were 0.2 M phosphate buffer and 0.025 M NaOH (in 60% sucrose), respectively. Running tap water, 15-18°, served as a coolant.

In all experiments described in this paper, the buffer was 0.0035 M sodium phosphate, pH 7.00 ± 0.02, and electrophoretic movement of the DNA was from the top to the bottom of the column. The voltage was in every case adjusted to keep the current at a constant value between 2.5 and 2.8 milliamperes, which corresponds to a voltage gradient of 0.7 to 0.8 volt per cm, sufficient to move the native DNA component approximately 33 cm from the origin in 48 hours. In other experiments, however, voltage gradients up to 1.8 volts per cm have been used successfully. The values of the voltage gradients given above are those calculated from the current applied and from the conductivity of the buffer without sucrose. Since the concentration of buffer was in every case constant throughout the gradient, the conductivity decreases several fold, and both voltage gradient and heat production correspondingly increase with increasing sucrose. The maximum heat production is, however, well within the safe upper limit suggested by Svensson. From electrophoretic runs of different duration it is calculated that the rate of movement of DNA is quite constant through the whole gradient, a result which indicates that the effect of sucrose in lowering the mobility of DNA (through increase of viscosity) approximately compensates for the corresponding increase in voltage gradient. The mobility of the native DNA component is calculated to be $25.5 \times 10^{-4}$ (cm$^2$ volt$^{-1}$ sec$^{-1}$), with a mean square deviation of 1.22 (based on 7 separate experiments); this value is in reasonable accordance with expectations from the results of boundary electrophoresis at comparable ionic strength but near 0° (4, 9).

Following electrophoresis, the column was emptied through rubber tubing into a fraction collector. Fractions were numbered from the bottom of the column. A volume of 300 ml corresponds to a distance of 40 cm along the chamber. The value of absorbance at 260 μm given for each fraction is that of the undiluted solution read against buffer and includes the absorbance of the sucrose, which was between 0.15 and 0.20. Only data on fractions containing detectable amounts is given in the figures; recovery of the added DNA was essentially quantitative. It was determined by control experiments that the properties of neither native, denatured, nor partially denatured DNA were changed by prolonged exposure to 60% sucrose.

Characterization of the various fractions in terms of native and denatured properties was based mainly on change of absorbance with temperature as described elsewhere (7). For this purpose, aliquots of the more concentrated fractions were diluted several fold with 0.005 to 0.01 M phosphate-10$^{-4}$ M EDTA, pH 7.3; the presence of 4 to 6% sucrose had only slight effect on the properties measured, but in all cases similar amounts of sucrose or appropriate amounts of baseline fractions were added to the comparison standards. Sucrose was removed from more dilute fractions by dialysis. Two indices were used; they were based on the information given in Fig. 2 of Reference 7. The first was based on the reversible increase of absorbance at 260 μm.
that occurs between 0 and 45°; expressed as $A_{450}/A_260$, the ratio is 1.007 for native DNA and, depending on the particular salt concentration used, between 1.15 and 1.21 for fully denatured DNA. The other index was based on the increase of absorbance that occurs on raising the temperature from 55° to 85°; expressed as $A_{260}/A_{550}$, the ratio is 1.02 to 1.04 for fully denatured DNA and 1.41 ± 0.02 for the various native samples. All values of absorbance were corrected for change of the specific volume of the solvent with temperature.

The thermal stability of the various samples of native DNA was determined in two ways. The first was from the complete absorbance-temperature curves such as those described by Marmur and Doty (10); the midpoints of these are designated as $T_m$. The second was from determination of the irreversible increase of absorbance that occurs upon brief exposure of the DNA to elevated temperatures followed by cooling (7, 11, 12); for convenience these curves will be called "irreversible thermal denaturation curves," and their midpoints designated as $T_e$.

The relative composition of the various fractions of DNA was estimated by the method of Fredericq, Oth, and Fontaine (13), which relates the composition of base-paired DNA to its ultraviolet absorption in acetic acid. For this analysis, samples were diluted several fold with acetic acid (final concentration, 0.1 M) and the ratio of absorbance at 260 mp to that at 280 mp ($A_{260}/A_{280}$, designated in the figures as $(A_{260}/A_{280})_{HAc}$), was determined. Our results with the procedure differ in some respects from those of Fredericq et al. (13). We find that considerable hypochromicity persists in 0.1 M acetic acid, and that the absorbance at 260 mp of DNA in this medium is only 34% above that of native DNA in neutral 0.1 M NaCl solution, rather than nearly 50%. We also find that the ratio $A_{260}/A_{280}$ for thymus DNA in acetic acid is somewhat below the value of 1.40 given by the corresponding mixture of nucleotides.

RESULTS AND DISCUSSION

Native DNA—Electrophoresis of native DNA alone always results in a single zone, as shown in Fig. 1. There is no evidence of fractionation within the zone either with respect to native properties or to composition. In addition to data given, values of $A_{260}/A_{280}$ were between 1.39 and 1.41 for all fractions. The absorbance-temperature and irreversible thermal denaturation curves of the peak fraction are given by Curves 1 and 5 of Fig. 2; these are essentially superimposable on corresponding curves for the original DNA; analogous curves for the other fractions are very close to these. These results also show that the DNA was in no way denatured by long exposure to the conditions used in the separation experiments.

Denatured DNA—Electrophoresis of fully denatured DNA also results in a single zone (Fig. 3), the mobility of which is about 8 to 10% less than that of native DNA. As with the native species, there is no evidence of fractionation within the zone. In addition to the data shown, values of $A_{260}/A_{280}$ for all the fractions were between 1.02 and 1.03. The complete absorbance-temperature curves were essentially the same as shown elsewhere (see Fig. 2 of Reference 7) for comparable conditions.
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FIG. 4. Density gradient electrophoresis of an artificial mixture of equal amounts of native- and heat-denatured DNA. Upper section, absorbance of the undiluted fractions at 260 mp. Lower section, O, A260:A280; •, (A260/A280)HAC. The volume of each fraction was 3 ml.

Artificial Mixtures—Electrophoresis of artificial mixtures of native and heat-denatured DNA yield patterns which are close to the summation of patterns given by the individual components. A typical example, using a mixture of equal parts of native and denatured DNA, is given in Fig. 4. The DNA of the leading zone (Fractions 32 to 35) is entirely native, while that of the trailing zone (Fractions 37 to 39) is completely denatured. Under the conditions employed, some overlapping of the zones occurs and a few per cent of the DNA which is always found in the trough fractions between the peaks (Fraction 36 for the experiment shown in Fig. 4) shows intermediate values of A260:A280 and of the other indices used to estimate extent of denaturation.

The values of A260:A280 are the same for all the fractions, an indication that the electrophoretic separation does not depend upon the composition of the individual molecules but only upon whether they are native or denatured.

Although not shown, data on the thermal stability of the various fractions may be summarized as follows. (a) Absorbance-temperature and irreversible thermal denaturation curves of Fractions 32 to 35 proved to be essentially the same as shown for native DNA by Curves 1 and 2 of Fig. 2. (b) Curves for Fractions 37 to 39 are the same as those given by totally denatured DNA. (c) The absorbance-temperature curve of the DNA in Fraction 36 proved to be biphasic and quite similar to the curves given by artificial mixtures of native and denatured DNA, which are also biphasic and close to the expected summation of the curves given by the individual components.

Partially Denatured DNA—In every case examined thus far, patterns of partially denatured DNA show a leading zone which contains native DNA and a trailing zone which contains denatured DNA; these two zones account for approximately 92 to 95% of the recovered material. Figs. 5 to 9 show typical examples, obtained with DNA that has been partially denatured to varying extents by heat, acid, or alkali.

By contrast with the results obtained with mixtures (cf. Fig. 4), the values of A260:A280 for the native fractions derived from partially denatured DNA are in every case lower than that of the original total DNA, while values for the denatured fractions are higher. Thus electrophoresis has effected a resolution of partially denatured DNA into a native fraction containing relatively high guanine plus cytosine and a denatured fraction containing relatively low guanine plus cytosine. It is estimated, from the curve given by Frederiq et al. (13), that the native and denatured fractions derived from 90% denatured DNA (Fig. 5) differ by about 9.6% in their content of guanine plus cytosine.

As with artificial mixtures, there is no evidence of fractionation within either the native or denatured zone.

In every case the native fractions separated from partially denatured DNA were more thermostable than the original un-
A typical example is shown in Fig. 2, which is a comparison of the absorbance-temperature and irreversible thermal denaturation curves of Fraction 25, derived from partially denatured DNA (50%; see Fig. 5), with corresponding curves for the undenatured DNA. Analogous curves for the other fractions of the native zone (Fractions 24 to 29) were essentially the same as shown for Fraction 25, while the curves obtained with DNA from the trailing zone (Fractions 31 to 35) proved to be the same as those for completely denatured DNA.

\[ \text{FIG. 7: Density gradient electrophoresis of DNA partially denatured (82\%) by heat. Upper section, absorbance of the undiluted fractions at 260 nm. Lower section, } O, A_{260}^\text{nm} A_{260}^\text{nm}; \bullet, (A_{260}^\text{nm}/A_{200}^\text{nm})\text{Ac. The volume of each fraction was 2.4 ml. The DNA was partially denatured by heating the solution (1.07 mg of DNA per ml, 0.001 M NaCl, 10^{-4} M EDTA; pH 7) at 61.7° for 15 minutes and then cooling it in an ice bath.} \]

\[ \text{FIG. 8: Density gradient electrophoresis of DNA partially denatured (60\%) by alkali. Upper section, absorbance of the undiluted fractions at 260 nm. Lower section, } O, A_{260}^\text{nm} A_{260}^\text{nm}; \bullet, (A_{260}^\text{nm}/A_{200}^\text{nm})\text{Ac. The volume of each fraction was 2.1 ml. The DNA was partially denatured by titrating a solution (0.98 mg of DNA per ml, 0.001 M NaCl; pH 6.8) to pH 11.89 at 25° with 0.01 M NaOH, and then neutralizing immediately by the addition of K_2HPO_4 and HCl.} \]

A few per cent of the DNA, which is only found in the trough fractions, shows intermediate values of the various indices. Absorbance-temperature curves of the DNA in these fractions were always biphatic and close to the expected summation of curves for (a) denatured DNA and (b) native DNA which is somewhat more thermostable than the original unfractionated DNA. In view of the evident overlapping of the zones it is likely that much of the material present in these fractions consists of fully native and fully denatured DNA molecules. However, the question of whether they also contain measurable amounts of partially denatured DNA molecules of the type described by Beer and Thomas (14) must await accumulation of sufficient material for further fractionation.

Our finding that, after removal of the denaturing conditions, well over 90% of the partially denatured DNA consists of fully native and fully denatured molecules is in agreement with the results of an examination of partially denatured DNA by electron microscopy (15). The fact that the native fractions derived from partially denatured DNA are in every case richer in guanine plus cytosine than the denatured fractions is in harmony with previous findings that, among deoxyribonucleates derived from different species, resistance to denaturation by either heat (10, 12), acid (16), or alkali (17) increases with the guanine plus cytosine content. It would appear that the hypothesis presented elsewhere (12) concerning heat denaturation may be applicable to acid and alkaline denaturation; that in all three cases denaturation becomes irreversible on dissociation of certain critical guanine plus cytosine-rich "nuclei"; and that this event occurs at higher temperature or greater degree of titration the higher the guanine plus cytosine content of the DNA molecule.

**SUMMARY**

The use of density gradient electrophoresis for separation of native and denatured deoxyribonucleate (DNA) is described. By this procedure, DNA partially denatured by heat, acid, or alkali has been resolved into two major fractions, one containing...
completely denatured DNA, and the other containing completely
native DNA. In every case, the denatured component is lower
in guanine plus cytosine than the original unfractionated DNA,
while the native component is higher. The native component
is more thermostable than the original unfractionated DNA with
respect to both reversible and irreversible denaturation.

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