The thermal denaturation of DNA: average length and composition of denatured areas

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ABSTRACT

A spectral study of melting curves of DNA ranging from 73 to 32% AT indicates that the base ratio of sequences melting within DNA are a linear function of temperature. A study of partially denatured DNA by electron microscopy, reversible renaturation and fractionation on hydroxylapatite suggests that the melting curve of DNA represents the melting of sequences which average 3-4 million daltons in length. These sequences appear to be a combination of two areas, one which is high in AT and denatures in the first three-quarters of the melting curve, and one which is high in GC and denatures in the final quarter. The length of these sequences appears to vary between 1.5-6 million daltons.

INTRODUCTION

Thermal denaturation curves of DNA are convenient to measure and are used to characterize physical states, base ratio and genetic homologies of DNA. The work reported in this paper outlines how, during the thermal denaturation of DNA, the average composition and length of denatured sequences vary with temperature and hyperchromic shift. The concentration of adenine plus thymine (AT) and guanine plus cytosine (GC) in denatured sequences is determined from ultraviolet absorption spectra. In the past, we have used the extinctions of nucleotides to approximate the extinction of the AT and GC pairs in denatured DNA (1), but further studies have shown that a proportionality exists in the extinctions between 250 and 280 nm. Consequently, our original measurements overestimate AT at the beginning and GC at the end of the melting curve. In this paper, concentrations are measured with empirically determined extinctions and the validity of the extinction is evaluated.

The first sequences to denature in DNA are AT rich and the last GC rich. We measure the association between these sequences by electron mi-

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croscopy of partially denatured DNA, hydroxylapatite chromatography of partially denatured DNA, and reversible renaturation of sheared DNA. The technique of reversible renaturation was developed by Geiduschek (2), who showed that denatured nucleotide pairs in thermally denatured DNA will renature when cooled if held in register by an undenatured region. Therefore, we use the extent of reversible renaturation of sheared DNA to determine the minimum length of DNA which includes an undenatured region. The variations in composition of the AT and GC rich regions is measured by fractionating partially denatured DNA on hydroxylapatite and determining the thermal stability and composition of the fractions.

MATERIALS AND METHODS

DNA Sources

DNA of Clostridium perfringens and calf thymus were purchased from the Worthington Biochemical Corporation (Freehold, N. J.). Bacterial strains from which DNA was isolated were purchased from the American Type Culture Collection (ATCC) (Rockville, Md.). Bacteria were cultured on nutrient agar slates and were grown in 3 liter cultures of 3% Bacto-Tryptone (Difco Laboratories, Detroit, Michigan) at the temperature recommended for the bacteria in the ATCC Catalog. The bacteria were harvested in late log phase, and the DNA was isolated according to the procedure of Marmur (3) with the addition of a phenol wash as recommended by Felsenfeld and Hirschman (4). DNA from C57BL/6J mouse liver was prepared in the same way. The DNA was stored as air-dried fibers at 4°. For use, the DNA was dissolved in the desired buffer and dialyzed for 24 hours against 100 volumes of the same buffer. The ATCC number, base ratio and phosphorous extinction coefficients of the bacterial DNAs are given in Table I.

TABLE I

Organism	ATCC number	%AT	eP
Clostridium perfringens	--	73.5	7,309
Bacillus natto	15245	57	6,195
Escherichia coli	11775	50	6,456
Serratia marcescens	13880	42.5	6,258
Sarcina lutea	381	32	6,268

Sources of DNA used in the spectral experiments. The base ratio is taken from Marmur and Doty (5).

Chemical Assays

DNA phosphorous was determined by the method of Bartlett (6). DNA concentration was determined by the diphenylamine reaction according to the method of Burton (7). Protein content of the DNA samples was measured by a modification of the biuret reaction described by Zamenhof (8).

Hydrolysis and Fractionation of DNA

Wheat seedling nuclease was purified according to the method of Hanson and Fairley (9) with the exception that chromatography on Bio-Gel was omitted. The enzyme hydrolyzes rRNA, 3'-nucleotides and denatured DNA. The enzyme behaves as an endonucleotidase on denatured DNA and has only a slight activity on native DNA. The pH optimum for the DNase activity is approximately 5.2.

For hydrolysis, DNA was dissolved in 0.05 M succinate buffer pH 6.0 and denatured at temperatures between 68-85°. Wheat nuclease was added to the solution and digestion was allowed to proceed at the denaturation temperature for one-half hour. Although the enzyme is relatively stable to heat (9), it is rapidly inactivated at 85°. Hydrolyzed and unhydrolyzed DNA were separated on hydroxylapatite (HAP) according to procedures outlined by Bernardi (10). Nucleotides were eluted in 0.05 M phosphate buffer, pH 6.8, and native DNA in 0.25 M phosphate buffer. Guanylic and adenylic acid were separated from other nucleotides on a 1x10 cm column of AG50 (Bio-Rad Laboratories, Richmond, Calif.), in 2 N and 3N HCl according to procedures outlined by Cohn (11).

Partially denatured DNA was fractionated on a 1x7 cm column of hydroxylapatite (DNA Grade, Bio-Rad Laboratories, Richmond, Calif.). Clostridium perfringens DNA, 0.2 mg, and E. coli DNA, 0.2 mg in a total volume of 4 ml of 0.015 M NaCl-0.0015 M sodium citrate were run onto the column and the column was washed with 0.18 M sodium phosphate buffer, pH 6.8 wich eluted approximately 20% of the DNA. The temperature of the column was raised by circulating water from a thermostatically controlled bath through an external jacket. The ultraviolet absorption of the effluent was continually monitored. No DNA was eluted between temperatures of 40 to 80°. Fractions were collected at 84, 88, 90, 94 and 99°. A final DNA fraction was eluted with 0.45 M phosphate buffer.

Electron Microscopy of Partially Denatured DNA

DNA was denatured at 50° for eighteen minutes in 0.02 M Na_2CO_3, 5 mM ethylenediamine-tetraacetate (EDTA), 10% formaldehyde, pH 11. This solvent is the same as that used by Wensink and Brown (12) except for the pH. DNA

was prepared for electron microscopy utilizing the formamide technique outlined by Davis et al.(13). DNA, 2 micrograms/ml in 60% formamide, 0.02 mg/ml lysozyme, 0.08 M Tris, 8 mM EDTA, pH 8.5, was spread on a hypophase of 30% formamide, 8 mM Tris, 0.8 mM EDTA. The DNA was picked up on formvar-coated grids, stained in $5x10^{-5}$ M uranyl chloride in 90% ethanol and dried in absolute ethanol. The grids were shadowed at an angle of 10 degrees with chromium and were photographed at a magnification of 6,400. Photographs were printed at an additional magnification of 5x. One micron of DNA length is taken to represent two million daltons, which is near the average observed under a variety of conditions of pH and solvent (14).

DNA Molecular Weight

The molecular weight of DNA was reduced by sonication of the solution with a Biosonic II sonic oscillator (Bronwill Scientific, Rochester, N. Y.). Chilled solutions were sheared from 5 to 120 seconds, depending on the molecular weight desired. For the longer shearing times, the solutions were permitted to rechill after 30 seconds of shearing.

The molecular weight of DNA in 0.2 to 0.3 M NaCl was determined by viscometry in a Contraves Low-Shear Rheometer Type LS100 (Contraves AG, Zurich) with a minimum shear rate of 0.07/sec. Calculations of molecular weight were made from the intrinsic viscosity reducted to zero shear by formulae given by Eigner (15).

The presence of nicks in DNA was measured by the kinetic formaldehyde technique of Lazurkin, et al. (16).

Melting Curves and Spectra

The absorbance of DNA solutions in 1 cm silica cells sealed with a Teflon stopper was measured in a Cary Model 16 dual-beam spectrophotometer. Solutions were heated by electric heating wires on the outside of the cell, and the solution temperature was monitored electronically with a thermistor probe. Prior to denaturation, DNA was degassed by vacuum. Absorbance was recorded either manually or by computer.

Reversible renaturation of DNA was measured by heating the DNA to a temperature within the denaturation range, recording the absorbance, cooling to a temperature 20 degrees below the Tm and again recording the absorbance. The cycle was repeated at increasingly higher denaturation temperatures until the DNA was fully denatured.

For recording spectra at a constant temperature, the temperature of the cell heating element was maintained by a YSI Proportional Temperature Controller (Yellow Springs Instrument Co., Yellow Springs, Ohio).

The composition of melting sequences in partially denatured DNA was analyzed with the hyperchromic spectral parameters of Hirschman and Felsenfeld (17).

Spectral ratios were calculated by dividing the absorbance at different wavelengths by the absorbance at 260 nm. This normalizes for differences in concentration.

The ratio of the extinction of the AT to GC pairs in 0.02 M Na_2CO_3, pH 11, 5 mM EDTA, 10% formaldehyde, the solvent used for electron microscopy, was determined from the hyperchromic spectrum (denatured spectrum minus undenatured spectrum) of E. coli and mouse DNA. The extinctions are based on the ratio the absorption at 270 (R_{270}) and 275 nm (R_{275}) to the absorption at 260 nm. At 275 nm, %AT = $-138.4 \cdot R_{275} + 232.7$, and at 270 nm %AT = $-163.6 \cdot R_{270} + 279.04$. A comparison of the calculated %AT at the two wavelengths provides an estimate or reliability.

As formaldehyde is volatile, end-absorbing, and undergoes a red shift when heated, we have found that the most reproducible method of collecting spectra is to use a water blank and to heat the DNA-formaldehyde solution directly in a spectrophotometer cuvette sealed with a Teflon stopper. For partial denaturation, the DNA is heated in a 50° water-bath for 1-5 minutes and, to stop denaturation, cooled to room temperature in a separate water bath which contains a small amount of detergent to keep the cuvette clean. After equilibrating at room temperature for five minutes, the cuvette is rinsed with distilled water, dried and the spectrum read at 260, 270 and 275 nm, where the readings show the greatest reproducibility. As the formula for base ratio depends on difference spectra, it is not necessary to include formaldehyde in the blank. The cycle of heating and cooling is repeated until the DNA is completely denatured.

The base ratio of thermally denatured DNA may be expressed in two ways. Spectra are taken at T_0, T_1, T_2, T_3, etc., where T_0 represents a temperature lower than that which causes denaturation and T_1, T_2, etc., are temperatures within the denaturation range. The base ratio may be calculated for sequences which melt from T_0 to T_1, T_1 to T_2, T_2 to T_3, etc., or as the base ratio of all melted sequences, i.e., from T_0 to T_1, T_0 to T_2, T_0 to T_3. This last will be called the total base ratio. As DNA approaches complete denaturation, the total base ratio approaches the average base ratio of the DNA.

RESULTS

The ultraviolet absorption spectrum of denatured DNA is greater than native DNA and has a different shape (18). As the spectrum varies with the

base ratio of the DNA, the average extinction of AT and GC pairs can be calculated from the spectra of DNAs of different composition (4,19,20). Of necessity, these extinctions are calculated from the spectrum of totally denatured DNA, but Fig. 1 shows that within the error of measurement, the spectrum of fully denatured DNA and the spectrum of partially denatured DNA are both related to temperature by the same function. Except for Clostridium DNA the relationship is linear. In our experience, Clostridium DNA frequently gives scattered spectra so the non-linearity can not be evaluated at present.

Marmur and Doty (5) have shown that the average denaturation temperature of DNA, the Tm, is a linear function of the base ratio of DNA. In unpublished experiments, we have analyzed the melting of DNA ranging from 73.5 to 32% AT using a graphical formula (1) and empirically determined extinctions for the AT and GC pairs at pH 4.8, 5.6, 6.8 and 10.4. At pH 4.8 and 5.6, the DNA was slowly degraded, but at all pH, the calculated change in base ratio with temperature fell around the Tm line, defined by Marmur and Doty's relationship. Fig. 2 shows the relationship between the Tm line and the base ratio of melting sequences in mouse DNA at pH 6.0 calculated with Hirschman and Felsenfeld's hyperchromic spectral parameters (17). The base ratios of melting sequences fall within 2-3 mole percent of the Tm line except at the extremes of 63-65° and 84°. These temperatures represent the ends of the melting curve where the absorbance change is too small to analyze accurately.

In order to compare the composition of denatured regions determined from spectra with their chemical composition, aliquots of calf thymus DNA in 0.05 M succinate buffer, pH 6.0 were brought to temperatures between 69 and 85°. The composition of denatured DNA in each sample was determined by Hirschman and Felsenfeld's hyperchromic spectral method. The denatured regions were then digested by wheat endonucleotidase. The digested material was separated from the undigested on HAP and its composition of the digest was determined by the method of Skidmore and Duggan (21). The adenine and guanine nucleotides were also separated on an AG50 column and the percent adenine determined. The results are shown in Fig. 3, which gives the total base ratio of the denatured DNA in each aliquot. Except at 66° where the denatured material was dilute, the spectral determination of the composition of denatured regions agrees with the direct determination within $\pm$2%.

The results show that the extinctions of the AT and GC pairs calculated from the spectrum of fully denatured DNA may be used to determine the composition of denatured sequences in partially denatured DNA. Except for an un-

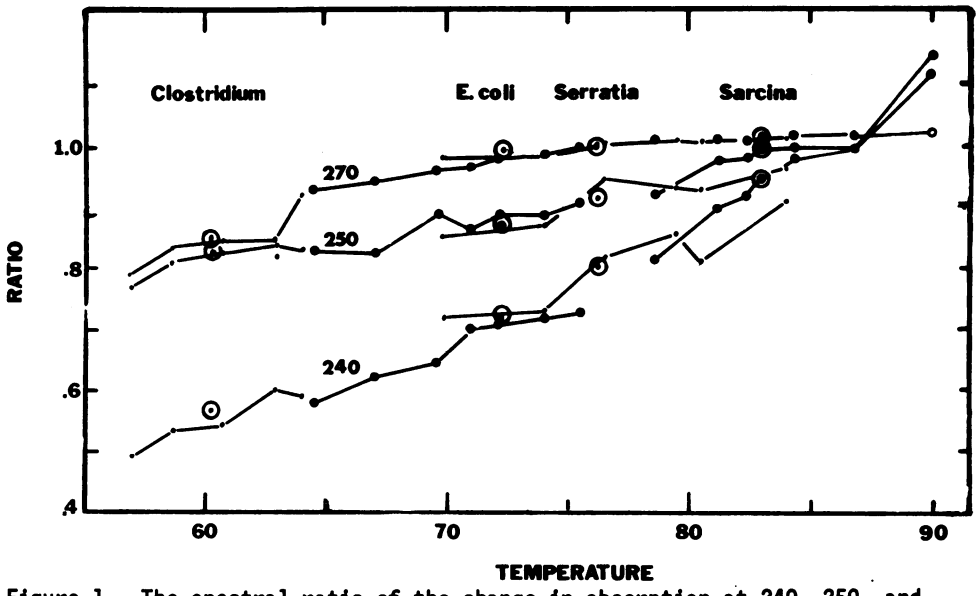

Figure 1. The spectral ratio of the change in absorption at 240, 250, and 270 nm to that at 260 nm measured during the denaturation of DNA at pH 6.8. The ratios for Clostridium and Serratia DNA are plotted with small points and the others with large. The large circules are the ratios for the hyperchromic spectrum of the totally denatured DNA plotted at their Tm.

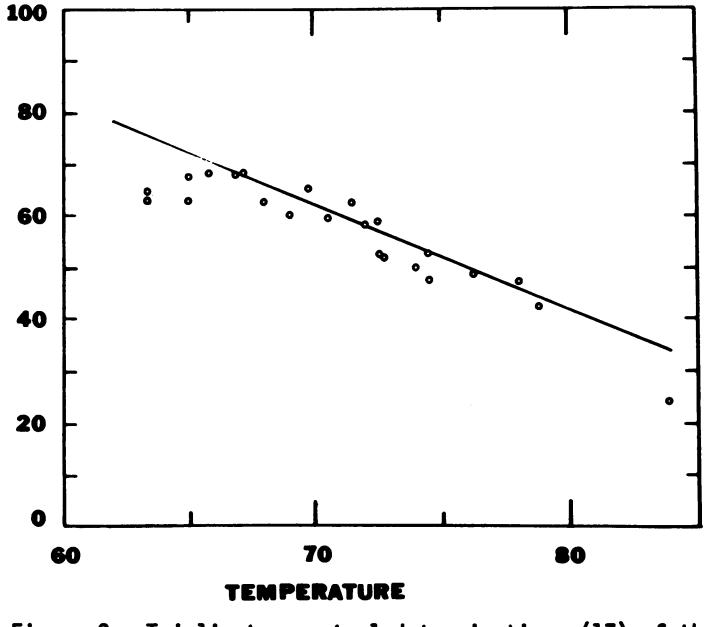

Figure 2. Triplicate spectral determinations (17) of the base ratio of sequences melting in calf thymus DNA during thermal denaturation in 0.05 M succinate buffer, pH 6.0. The diagonal line represents the relationship between Tm and base ratio of DNA in this solvent.

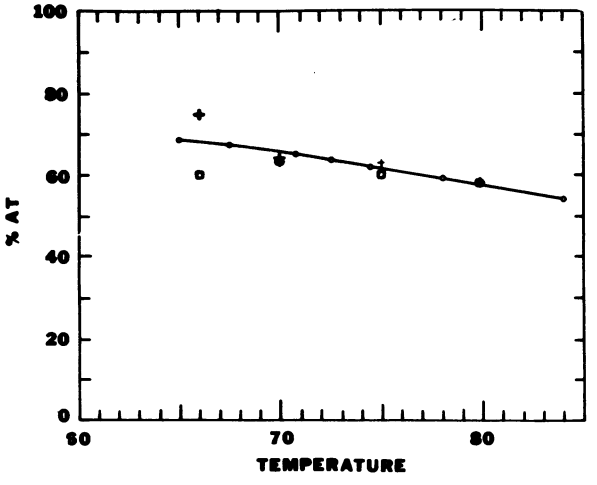

Figure 3. The comparative determination of the total base ratio of single-stranded regions in calf thymus DNA during thermal denaturation in 0.05 M succinate buffer, pH 6.0. The connected small circles represent the spectral determination. The composition of nucleotides released from the denatured regions by wheat nucleotidase was determined by the method of Skidmore and Duggan (21) (crosses) and fractionation (squares).

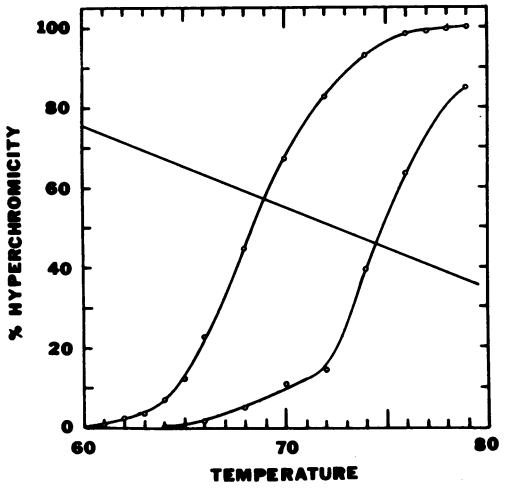

Figure 4. The denaturation and reversible renaturation of mouse DNA in 0.03 M NaCl-0.003 M sodium citrate, monitored at 260 nm. The curve to the left represents the hyperchromic shift, normalized to 100% of the DNA during heating. The curve to the right represents the absorption of the DNA after cooling from the different denaturation temperatures to 55°. This curve, the reversible renaturation curve, has been normalized to the maximum hyperchromicity of the denatured DNA. The diagonal line represents the base ratio in %AT of melted sequences and applies to the denaturation curve. The base ratio can be read on the ordinate.

certainty at the very beginning and end of the melting curve, the composition of melting sequences are a linear function of temperature. This function is the same as Marmur and Doty's relationship between Tm and base ratio (5), with the constants adjusted for the solvent. Consequently, their relationship may be used to estimate the composition of sequences melting in the different parts of the thermal denaturation curve of DNA.

The thermal denaturation curve of DNA is composed of three sections: an initial AT rich section which is concave upwards, a mid section which is approximately linear, and a terminal GC rich section which is concave downwards. The approximate composition of sequences in mouse DNA shown in Fig. 4 range from 75-62% AT in the AT rich section, 62-52% AT in the mid section, and 52-40% AT in the GC rich section. In DNA of 10-20 million daltons, these sections may occur on the same strand or on separate strands. How they occur determines the physical properties of DNA and reflects the composition of codons.

The most direct technique for visualizing the distribution of the regions among strands is by electron microscopy of partially denatured DNA. However, in the method, formaldehyde is used to prevent reversible reassociation of the partially denatured strands and formaldehyde is a denaturing agent. Consequently, to utilize electron microscopy for this study, it is necessary to show that both heat and formaldehyde denature sequences of the same composition.

The composition of sequences denatured by formaldehyde was measured with absorption spectra. The extinctions of the AT and GC pairs was determined in 10% formaldehyde, 5 mM EDTA, 0.02 M Na_2CO_3, pH 11, as outlined in Methods. In this solvent, denaturation is not observed at 25°, but progresses to completion in about one half hour at 50°. Mouse DNA, in this solvent, was placed in a sealed cuvette, heated 3-5 minutes at 50°, cooled to room temperature and the absorption spectrum measured. The heating and cooling cylce was repeated until the DNA was fully denatured. The total base ratio was calculated from the spectra and is shown plotted against the percent hyperchromicity at 260 nm in Fig. 5. Such a plot mimics a thermal denaturation curve because the base ratio is a linear function of temperature, so may be used in place of temperature.

The denaturation of DNA with formaldehyde approximates the denaturation of DNA with heat: the first sequences to melt are rich in AT, and the last, in GC. The hyperchromic shift is sigmoid and the total base ratio converges on the base ratio of the DNA at the end of the melting curve similar to ther-

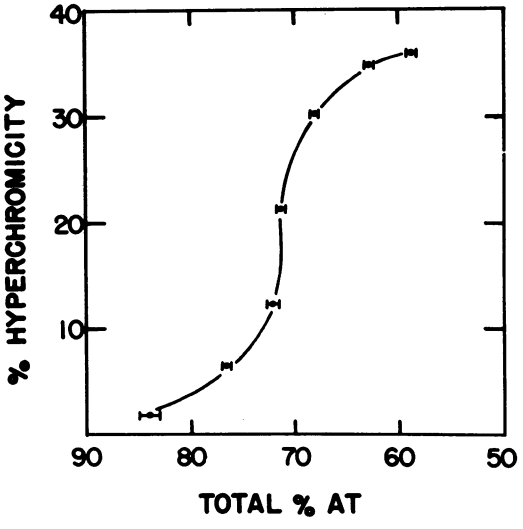

Figure 5. The base ratio versus the hyperchromic shift of mouse DNA in 10% formaldehyde, pH 11.0.

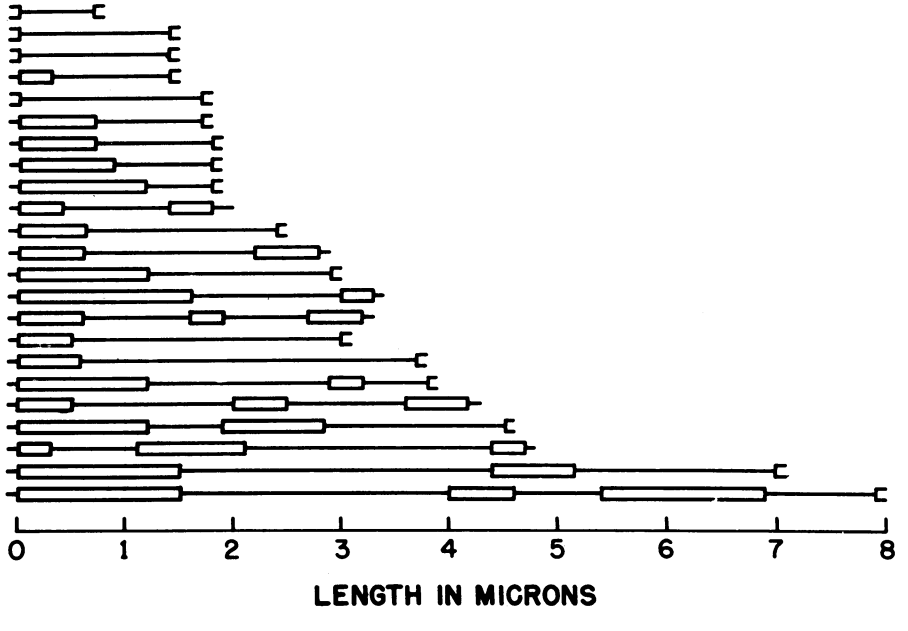

Figure 6. Partially denatured mouse DNA observed in electron micrographs. Denatured loops are represented by rectangles and undenatured areas by lines.

mal denaturation. However, the first sequences to melt are about 85% AT, while with thermal denaturation they are about 75% AT. This indicates less involvement of GC pairs in the initial formaldehyde reaction and would lead to shorter denatured regions. In the later stages of the formaldehyde reaction, GC is also denatured so the length of denatured regions should approach those obtained with thermal denaturation.

A sample of mouse DNA was brought to 54% hyperchromic shift at 260 nm in the formaldehyde solvent. From spectra, the total base ratio of denatured regions was calculated as 72% AT with a range of 88-55% AT. DNA was prepared for electron microscopy by the procedure outlined in Methods.

The DNA, as measured from the micrographs, is shown in Fig. 6. The denatured loops measure 0.87 ± 0.55 microns and the undenatured regions, 1.41 ± 0.75 microns. Denatured and undenatured regions alternate with an average periodicity of 2.28 microns, which corresponds to approximately 4.5 million daltons.

While the DNA went through a 54% hyperchromic shift, denatured loops are less than half the total length. This may result from overestimating the extent of denaturation at 260 nm, partial reversible renaturation of strands during preparation of the DNA for electron microscopy, or breakage of denatured loops. However, from the spectral studies, the denatured areas include AT rich sequences and a proportion of the sequences which melt in the mid part of the melting curve. These areas alternate with undenatured areas which are more GC rich.

The alternation of AT and GC rich areas may be further evaluated with a method developed by Geiduschek (2), who showed that partially heat denatured DNA will reversibly renature when cooled, provided the denatured nucleotide pairs are held in register by an undenatured region. A reversible renaturation curve of mouse DNA is shown in Fig. 4. The DNA reversibly renatures nearly completely until it is 80% denatured. Beyond 80% hyperchromic shift, where GC rich regions become denatured, reversibility of renaturation is lost. Thus, nearly all unsheared strands of mouse DNA, 25 million daltons, contain at least one undenatured GC rich region at 80% hyperchromic shift. To determine whether the strands contain more than one GC rich region, they are sheared to shorter sizes, denatured and reversibly renatured. From the length of denatured loops in the micrographs, the DNA would have to be sheared to a size less than 3 million daltons for any loops to be sheared free of undenatured ends.

Figs. 7 and 8 show the percent reversible renaturation versus shear length of mouse and B. natto DNA at different stages of denaturation. At

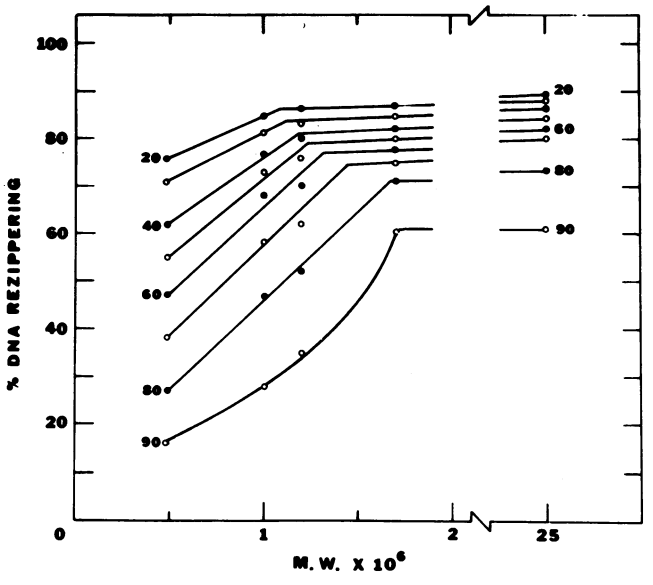

Figure 7. The relationship between reversible renaturation (rezippering) after partial denaturation of mouse DNA in 0.03 M NaCl-0.003 M sodium citrate and molecular weight. The extent of denaturation of each sample has been indicated for alternate lines and the points for alternate lines have been filled in. The abscissa has been compressed between 2 and 25 million daltons.

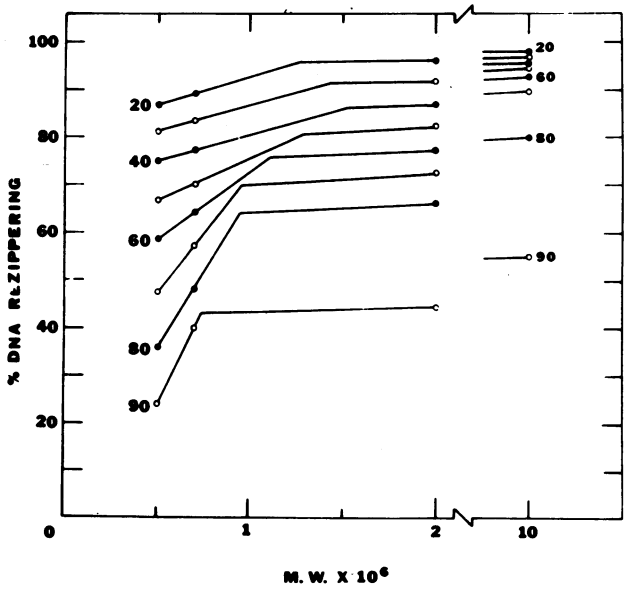

Figure 8. The reversible renaturation of B. natto DNA. See the legend to Fig. 7 for details.

20% hyperchromic shift, unsheared B. natto DNA renatures nearly completely, but mouse DNA does not. The difference does not appear to be due to nicks in mouse DNA because neither DNA contained measurable nicks when assayed by the kinetic formaldehyde method (16) (limit of detectability, 1 nick per 6 million daltons). Either there are AT rich strands in DNA 25 million daltons long, or, as Hoff and Roos suggest (22), there is a limit to the length of denatured regions which can reversibly renature. B. natto DNA sheared to 2 million daltons renatures less well than mouse DNA sheared to 1.7 million daltons. If this difference is not an artefact, then denatured loops may be longer in B. natto DNA than in mouse DNA. Mouse DNA renatures nearly as well at a shear size of 1.7 million as at 25 million daltons. At 80% hyperchromic shift, at which the GC rich regions are undenatured, 70% of the fragments reversibly renature. This figure is increased to 80% if corrected for sequences which do not renature in unsheared DNA. Thus, at 80% hyperchromic shift, most denatured regions, sheared to 1.7 million daltons, contain an undenatured region. The reversible renaturation curves will be analyzed in more detail in the discussion, but they support the observation from the micrographs that AT and GC rich areas occur within a few million daltons of each other.

The AT and GC rich regions both represent sequences of a large variety of compositions. The variation can be measured by fractionating DNA into separate groupings of AT and GC rich regions. The micrographs of partially denatured DNA show that undenatured areas differ in length from 1 to 3 microns. This difference suggests that it may be possible to elute partially heat denatured DNA from HAP on the basis of the length of undenatured GC rich regions. Under the conditions of partial denaturation, denatured regions would reversibly renature when the fraction is cooled. As denatured DNA is eluted from HAP by 0.15 M phosphate buffer, and unsheared native DNA by 0.2 M and above (10), we chose 0.18 M sodium phosphate buffer, pH 6.8, to elute DNA that is partially denatured. Preliminary experiments with calf DNA showed that while fractionation appears to take place, the base ratio of the fractionated DNA does not necessarily become enriched with GC as the elution temperature increases. Therefore, to better determine whether fractionation takes place on the basis of GC rich regions, Clostridium DNA (72% AT) and E. coli DNA (50% AT) were mixed, placed on HAP column and thermally eluted at temperatures between 84 and 99°. The combined melting temperatures of the DNA samples range from 80 to above 100° in 0.18 M phosphate buffer. DNA not eluted at 99° was removed with 0.45 M phosphate buffer, pH 6.8. The fractions

were thermally denatured and the base ratios were measured from spectra with Hirschman and Felsenfeld's hyperchromic spectral parameters (17).

The results, presented as the change in hyperchromic shift with temperature, are shown in Fig. 9. The fractions contain native and, as well, denatured DNA. The 84° fraction is slightly more GC rich than the 88° fraction, which may reflect overloading the column. At 88° and above, the fractions become enriched with GC proportionally to the temperature and the differential melting curves suggest that the melting temperature of the most GC rich sequences of Clostridium and E. coli DNA also increases. The concentration of Clostridium DNA decreases with temperature while the concentration of E. coli DNA increases. Thus, fractionation of partially denatured DNA according to the base composition of undenatured regions appears to take place on HAP in 0.18 M phosphate buffer.

We have assumed that partially denatured DNA fractionated on HAP will reversibly renature when cooled, but the fractions all contain denatured DNA. Except for the 88° fraction, the hyperchromic shifts shown in Fig. 9 are less than 50% of the total. DNA will reversibly renature in phosphate buffer. Therefore, the presence of denatured DNA may be due to the wide temperature jumps between the fraction, thermal scission of the DNA, or possibly the hydroxylapatite, as calcium compounds dissolved from it might interfere in reversible renaturation.

The width of the melting curves of each DNA in the fractions covers approximately 12°, while the width of the curves of unfractionated DNA covers 23-25°. The most GC rich sequences in E. coli DNA vary from about 33% AT in the 90° fraction to 20% AT in the 0.45 M fraction, while the most AT rich fraction varies from 50% AT in the 94° fraction, to 40% in the 0.45 M fraction. Therefore, the base ratio of the AT and GC rich areas vary at least 10% between all fractions. As the most AT rich area of unfractionated E. coli DNA has a thermal stability which corresponds to 70% AT, the possible base ratio variation of the AT rich region may be greater than 30%.

DISCUSSION

At 80% hyperchromic shift, 70% of mouse DNA fragments sheared to 1.7 million daltons reversibly renatures. Therefore, the fragments are composed of an undenatured GC rich region and a denatured region which is more rich in AT. The average DNA length which includes both these regions may be estimated from the reversible renaturation curves.

Let L be the length which includes both these regions. When DNA is partially denatured, the average length of the denatured region will be assumed

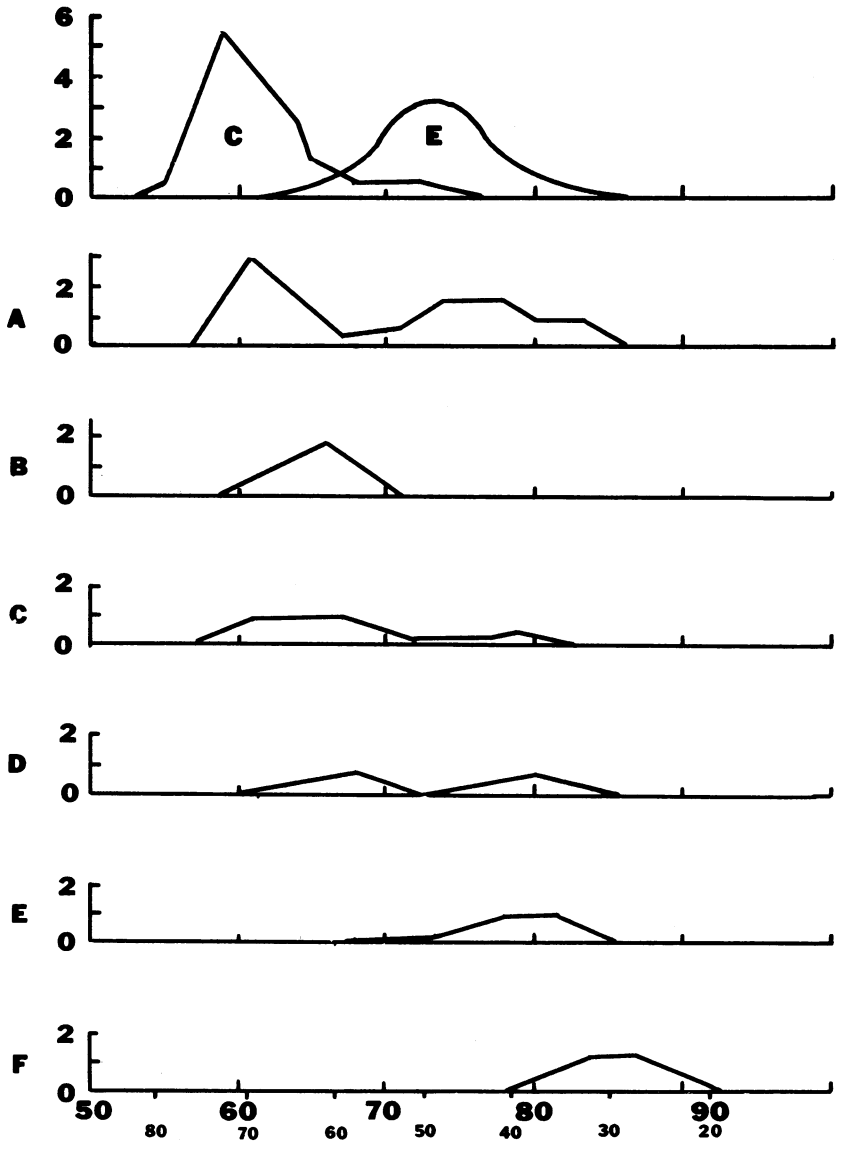

Figure 9. Fractionated DNA. The change in percent hyperchromicity of DNA in 0.01 M NaCl-0.001 M phosphate buffer, pH 6.8, plotted versus the denaturation temperature. The top figure shows the melting curves of unfractionated Clostridum (C) and E. coli (E) DNA. The total hyperchromic shift of these samples was 38% and 33% respectively. Figures A-F are the melting curves of these mixed DNAs fractionated on hydroxylapatite. The elution temperature, base ratio and hyperchromic shift (H) of the fractions are: A, 84°, 57% AT, 34.5% H; B, 88°, 64% AT, 16.5% H; C, 90°, 63% AT, 15% H; D, 94°, 58% AT, 11% H; E, 99°, 45% AT, 13% H; F, DNA eluted with 0.45 M buffer at 99°, 35% AT, 15% H. The large numbers on the abscissa are the temperature and the small numbers the base ratio in %AT of sequences which melt at these temperatures.

to be LH, where H is the fractional hyperchromic shift normalized to one.

Shearing the loop will produce a set of fragments, some of which will include an undenatured region, and will reversibly renature. The longest denatured fragment which will reversibly renature is equal in length to the shear size, MWs, minus the length of the undenatured region, which, for the present, will be considered to be equal to zero. The minimum length of a denatured area which will reversibly renature approaches zero, so the approximate range of lengths which renature is from MWs to zero. Consequently, the average renaturable length of a fragment which represents one end of a denatured loop is MWs/2 and the average for both ends is MWs.

As MWs is the approximate length of the sheared loop which can renature, LH-MWs is the length which will not renature. The fraction, U, of the sheared DNA which will not renature is this length divided by L, or U=(LH-MWs)/L. Rearranging terms, L=MWs/H-U. To rewrite the formula in terms of reversible renaturation, U is set equal to 1-R, where R is the fraction of the DNA which renatures. This substitution for U leads to L=MWs/H+R-1, the form of the formula we have used.

The formula predicts that L is constant throughout the denaturation range. However, the actual range of loop lengths which will not renature is LH-MWs$^{\pm}$MWs, but the variation is disregarded in the formula, so L can only assume a constant value when the average denatured loop length is greater than MWs. In the early stages of denaturation, U is greater than zero and L will be overestimated. Loop lengths shorter than the minimum shear size can not be measured by reversible renaturation experiments. If shearing leads to denaturation of fragments, U will be disproportionately large at the lower shear sizes. Thus, L would appear to increase as the shear size decreases, because L is inversely proportional to H-U.

The lengths of denatured loops calculated according to L=MWs/H+R-1 versus the percent hyperchromic shift is shown in Fig. 10. The values of R were taken from Figs. 7 and 8 for mouse and B. natto DNA. Ten percent has been added to all the values of R of mouse DNA to compensate for sequences which do not renature in the unsheared DNA.

In both DNA samples, the calculated value of L is greatest in the initial stages of denaturation, which reflects the error in the formula which occurs when LH is close to MWs. The greater length of L calculated from the reversible renaturation curve of B. natto DNA sheared to 2 million daltons may either result from incorrect measurements, or the above error. As the values of L calculated at 0.5 million daltons are near those calculated between 0.7

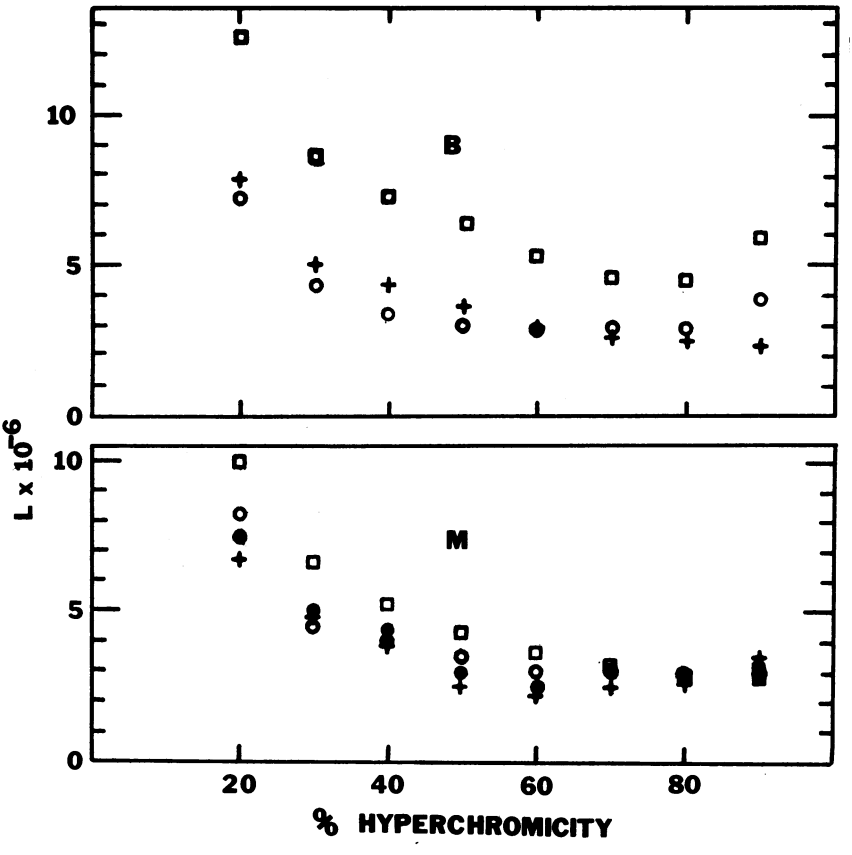

Figure 10. The calculated values of the period length, L, in millions of daltons for B. natto DNA (B) and mouse DNA (M) plotted versus the hyperchromic shift. The different points represent different shear sizes; in million daltons, the shear sizes are: B. natto DNA, squares, 2.0; crosses, 0.7; and circles, 0.5. Mouse DNA, squares, 1.7; black circles, 1.2; crosses, 1.0; and circles, 0.5.

to 1.7 million daltons, thermal destabilization of sequences by shearing is not a measurable variable in these experiments. At these shear sizes, L approaches a constant value near 3 million daltons beyond 40% hyperchromic shift. Therefore, if the model for reversible renaturation contained in the formula is correct, then Fig. 10 shows that AT, intermediate, and GC rich sequences occur in DNA with a periodicity of 3 million daltons.

In the formula used to determine L, the length of undenatured ends of sheared fragments and variations of loop length were disregarded. The error induced by disregarding the ends is small compared with L. Taking K to be equal to the minimum stable lengths of the end, L=(MWs/H+R-1)-(K/H+R-1). R for mouse DNA at 80% hyperchromic shift lies between 0.08 and 0.18 (Fig. 7), so K ranges between 0.1 and 0.3 million daltons. K can be calculated from the denaturation curve in Fig. 4 (23). At 80% hyperchromic shift, the Tm is 72°, the composition of melting sequences is 48% GC, and the molarity is 0.033. The minimum stable length is, therefore, 0.1 million daltons. The error in disregarding the variation in loop length is greater than disregarding K. The formula for L should be L=(MWs±MWs)/H-R-1, which implies a variation in L of plus or minus one hundred percent. However, the length distribution is not known. If the standard deviation of measurements from the micrographs are used (±57%), then L=3±1.7 million daltons.

The model for DNA melting implied by the formula is extremely simplified compared to analyses of melting by thermodynamic calculations. According to Lazurkin, et al. (16), loop length is not a linear function of the hyperchromic shift. Their analysis suggests that at 10-20% hyperchromic shift, helical regions have a length equal to between .6 to 1.5 million daltons and at 50% hyperchromic shift, about 0.3 million daltons, whereas our linear extrapolation would suggest lengths of 2.4 and 1.5 million daltons at these hyperchromicities. However, our analysis only relates to loops that are long enough to be sheared and are visible in our micrographs.

Everson, Mego and Taylor (24) have observed a prominent periodicity of loops at 1.9-2 microns in micrographs of partially denatured hamster and chick DNA, and a minor periodicity at 0.4-0.5, 0.8, and 1.1-1.2 microns. Wensink and Brown (12) have investigated the denaturation of ribosomal DNA of Xenopus laevis by electron microscopy. The denaturation pattern of this DNA has a 8.7±0.6 million dalton repeat which is divided into two parts, one of 5 million daltons which is 60% GC, and one of 3 million daltons which is 73% GC. The spacing between loops and undenatured areas in this DNA support the measurements of periodicity we made from the reversible renaturation

curves, but the composition of denatured and undenatured areas within the average period appears to exhibit a wide variation.

To mention other aspects of the experiments, W. Kroeker (25) has shown that at pH 5 and 22°, wheat nuclease hydrolyzes native poly d(A-T), but not poly dG-dC. Under these conditions, the enzyme hydrolyzes E. coli and viral DNA to a limit digest of 2.5-3 million daltons, with the release of 8 nucleotide equivalents per limit polymer. Our samples, at pH 6, heated to a temperature at which thermal denaturation begins and digested with wheat nuclease were reduced to fragments between 1.5-2 million daltons. The fragments would not reversibly renature, indicating that they were nicked. It is interesting to note that if DNA is partially denatured and treated with nucleotidase to digest denatured regions, after digestion stops, the temperature must be elevated 1.5-2° before further digestion takes place. Apparently, the hydrolysis, by creating new ends in contact with solvent, denatures the DNA so that hydrolysis continues along the fragment until a GC rich area is encountered.

As to the fractionation of partially denatured DNA, Wilson and Thomas (23) have shown that short DNA duplexes are eluted from HAP columns at lower phosphate buffer molarities than long duplexes. Presumably, the fractionation we observe with 0.18 M sodium phosphate buffer is due to length variations in the undenatured regions. The large initial peak at 84° shown in Fig. 9 may indicate that the column was overloaded, although no DNA was eluted at 80°. Calf DNA, sheared to 0.5 million daltons, which represents a length of about 800 nucleotide pair, is eluted from the column by 0.18 M sodium phosphate buffer at room temperature. According to the data of Wilson and Thomas, such fragments should not have eluted at molarities below 0.2. The difference may be due to our use of commercially prepared HAP and higher concentrations of DNA.

Our conclusion from all the experiments is that the denaturation curve of DNA represents the melting of sequences which average 3-4 million daltons in length. The sequences appear to be a combination of two areas, one which is more AT rich and melts in the first three quarters of the denaturation curve, and one which is more GC rich and melts in the final quarter. The length of these regions appears to vary between 1.5 and 6 million daltons and at least 10% in their base ratio.

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