HEAT DENATURATION STUDIES OF RAT LIVER MITOCHONDRIAL DNA

A Denaturation Map and Changes in Molecular Configurations

DAVID R. WOLSTENHOLME, ROBERT G. KIRSCHNER, and NICHOLAS J. GROSS

From the Division of Biology, Kansas State University, Manhattan, Kansas 66502; and the Whitman Laboratory, Department of Biology and the Departments of Pathology and Medicine, University of Chicago, Chicago, Illinois 60637. Dr. Wolstenholme's present address is the Department of Biology, University of Utah, Salt Lake City, Utah 84112. Dr. Kirschner's present address is the Department of Pathology, United States Public Health Service Hospital, Baltimore, Maryland 21211. Dr. Gross's present address is Kew Richmond, Surrey, England.

ABSTRACT

The effect of progressive denaturation of open circular molecules (component II) and supercoiled covalently closed circular molecules (component I) of rat liver mitochondrial DNA has been followed by heating in the presence of formaldehyde and examination in the electron microscope. After heating at 49°C, two, three, or four regions of strand separation were visible in 25% of the component II molecules. Comparisons of the patterns of distribution of these regions in individual molecules indicated that they occurred at at least three specific positions around the molecule. Also, these regions, which were assumed to be rich in adenine and thymine, were within a segment which was less than 50% of the length of the molecule. After heating at 50°C, up to 14 regions of strand separation were observed, but when comparisons were made no clear groupings were found. At 51°C, component II molecules were completely separated into a single-stranded circle and a single-stranded linear piece of similar length. Strand separation was accompanied by shortening of the molecule. At 70°C, single-stranded circles had a mean length of 2.7 μ, compared with 5.0 μ for native molecules. Progressive heating of component I molecules resulted first in conversion to an open circle (I′) and then to a second supercoiled form (I″). Visualization of further denaturation products of component I was prevented by cross-linking of the molecule by formaldehyde at high temperatures.

INTRODUCTION

Mitochondrial DNA (mtDNA) of all metazoan animal species examined, from nematode worms to man, is in the form of circular molecules with contour lengths of about 5 μ (for recent reviews see Swift and Wolstenholme, 1969; Wolstenholme et al., 1971). There is no evidence for more than one size class of circle within a single species. Also, for any one species, the mtDNA forms a single band upon equilibrium centrifugation in cesium chloride. These observations, and the results of studies of renaturation kinetics of chick mtDNA (Borst, Ruttenberg, and Kroon, 1967) are consistent with there being only one kind of circle with respect to base sequences. This would

imply that the maximum genetic information which the mtDNA of any one animal species can carry is defined by the length ($\simeq 5\ \mu$) of a single circle of DNA.

In an attempt to test this further, and to gain information concerning the gross arrangement of base sequences in individual molecules of mtDNA from rat liver, we have used the denaturation mapping technique of Inman (1966; 1967). This technique is based upon the finding that regions rich in adenine and thymine melt reproducibly in different copies of a DNA molecule at temperatures lower than regions rich in guanine and cytosine (Inman, 1966; Follett and Crawford, 1967 *b*, 1968). By heating the DNA to different temperatures in the presence of formaldehyde, which prevents the rejoining of separated bases upon cooling (Freifelder and Davison, 1962; Inman, 1966), and examining the DNA in the electron microscope, regions of strand separation can be detected and individual molecules mapped with respect to the gross distribution of the different base pairs.

For human papilloma virus DNA and polyoma virus DNA, it has been shown by electron microscopy that partial denaturation by heating in the presence of formaldehyde affects the tertiary structure of the supercoiled covalently closed circular form of the molecule (Follett and Crawford, 1967 *a*, *b*; 1968). The present report also describes changes in molecular configuration of covalently closed circular mtDNA molecules, as well as open circular mtDNA molecules, upon progressive heating.

MATERIALS AND METHODS

Preparation of Mitochondria

Mitochondria were prepared from liver of adult rats (weighing 100–200 g each) following the method described by Kirschner et al. (1968). Livers were removed from rats sacrificed by cranial fracture, and immersed in 10 vol of 0.3 M sucrose, 1 mM disodium ethylenediaminetetraacetate (EDTA), and 0.01 M Tris·HCl (pH 7.4) at 0°–4°C, homogenized first in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) (top speed 10 sec), and then in a Potter-Elvehjem homogenizer (top speed, 2 strokes) with a fitted Teflon pestle, and centrifuged at 480 *g* for 10 min. The supernatant was further centrifuged at 7710 *g* for 12 min and the resulting pellet suspended in the aforementioned buffer. Magnesium chloride was added to 0.007 M and pancreatic deoxyribonuclease (DNase) (Worthington Biochemical Corp., Freehold, N.J.) to 100 μg/ml, and the suspension incubated at 37°C for 30 min (Rabinowitz et al., 1965). DNase digestion was stopped by adding EDTA (pH 8.0) to 0.04 M, and the DNase removed by alternately centrifuging out and resuspending the mitochondria 3 times in 0.3 M sucrose, 0.01 M Tris·HCl, and 0.04 M EDTA, (pH 7.4). The mitochondria were finally suspended in 0.15 M NaCl, 0.1 M EDTA, and 0.05 M sodium phosphate at pH 8.5 and frozen.

DNA Extraction and Purification

Suspensions of mitochondria were thawed and then lysed with 2% sodium dodecyl sulfate, and DNA was extracted by the method described by Kirschner et al. (1968). The lysate was shaken twice for 60 min at room temperature (about 20°C) with phenol equilibrated with the same buffer in which the mitochondria were suspended. Phenol was removed from the aqueous phase by shaking with ether. The ether was blown off with N_2 gas. Ribonuclease (Worthington Biochemical Corp., previously heated to 90°C for 5 min at pH 5.0) was added to 200 μg/ml, and the mixture incubated at 37°C for 30 min. The solution was dialyzed against 0.35 M NaCl, 2 mM EDTA, and 0.05 M sodium phosphate at pH 6.7 for 2 hr with two changes and then applied to a methylated albumin kieselguhr column (Mandell and Hershey, 1960). The column was washed with 0.4 M NaCl until no UV-absorbing material appeared in the effluent, and the DNA was then eluted with 0.8 M NaCl. Both of the aforementioned saline solutions contained 0.05 M sodium phosphate.

DNA was prepared from whole liver by a similar procedure.

Thermal Denaturation

FOR ELECTRON MICROSCOPY: 20-μl samples of solution containing approximately 0.2 μg DNA, 0.05 M phosphate (pH 7.8), and 10% formaldehyde were made up and sealed in 100-μl capillaries. Before use, the formaldehyde was treated as follows: to 10 ml of 37% formaldehyde, 0.1 ml 1 N NaOH was added, and the solution was heated to 100°C for 10 min in a sealed vessel. The formaldehyde was finally adjusted to pH 7.8 with NaOH, and sodium phosphate at pH 7.8 was added to 0.05 M (Freifelder and Davison, 1963).

Some capillaries were individually heated at specific temperatures for 10 min in a thermostatically controlled water bath and then cooled in ice water. Samples in other capillaries were not heated.

MELTING CURVES: Thermal denaturation of DNA in 0.05 M sodium phosphate or 0.05 M sodium phosphate and 10% formaldehyde was followed by

observing changes in hyperchromicity in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with dual thermoplates connected to a Haake heating unit (Chas. F. Haake, Seaford, N. Y.). The DNA solutions, at a concentration of 10–15 μg/ml, were overlayed with mineral oil and continuously heated at the rate of 0.5°C/min in 0.7 ml quartz cuvettes. The temperature was measured with a thermocouple placed in the same compartment as the sample.

Electron Microscopy

DNA was prepared for electron microscopy by the protein monolayer technique following the method described by Freifelder and Kleinschmidt (1965). The contents of a capillary were mixed with 75 μl of 1 M ammonium acetate and 5 μl 1% cytochrome *c*. The solution was then allowed to flow down an inclined glass slide onto a hypophase of 0.3 M ammonium acetate containing 0.5% formaldehyde. The surface film was left standing for 10 min and then picked up on carbon-coated Formvar films supported on 100-mesh copper grids. The grids were shadowed on a rotary turntable at an angle of about 8° with 8 mg of plantinum-palladium wire (80:20), evaporated from a 23 mil tungsten wire at a mean distance of 10 cm from the grids, in a Kinney model KSE-2 evaporating unit (Kinney Vacuum Co., Boston, Mass.). The specimens were examined and photographed (using projector pole piece 2) with an Hitachi HU-11B electron microscope at an original magnification of × 11,000. The magnification was calibrated with a diffraction grating replica (2160 lines/mm). Measurements of shadowed molecules were made on positive prints. Molecules including strand separations were measured with a Keuffel and Esser model No. 62-0335 map measure (Keuffel and Esser Co., Morristown, N.J.) (accurate to better than 2 mm per measurement) at a magnification of × 150,000. All other molecules were measured with a Keuffel and Esser model No. 62-0300 map measure (accurate to better than 1 cm per measurement) at a magnification of × 100,000.

Estimates were made of the percentage weight of DNA in the double-stranded and single-stranded forms in each preparation heated at 51°C and above, by examining samples of 300 molecules. A single-stranded circle was assumed to be one-half the weight of a double-stranded circle. The amount of DNA in the single-stranded linear form was calculated by measuring the linear molecules in each sample and dividing the total length by the mean length of the single-stranded circular molecules of the same sample.

In the sample heated at 49°C, some of the open circular molecules had one or a low number of crossovers of the filament. For the purpose of mapping the positions of strand separations, the following rules were observed, following Inman (1966). When a strand separation occurred on each side of a point of crossover, the relationship of the strand separations to each other with respect to distance along the molecule was only considered unambiguous when the filaments crossed approximately at right angles (see Fig. 3). When the strands crossed at a narrow angle or ran parallel to each other, then the molecule was not used to make comparisons.

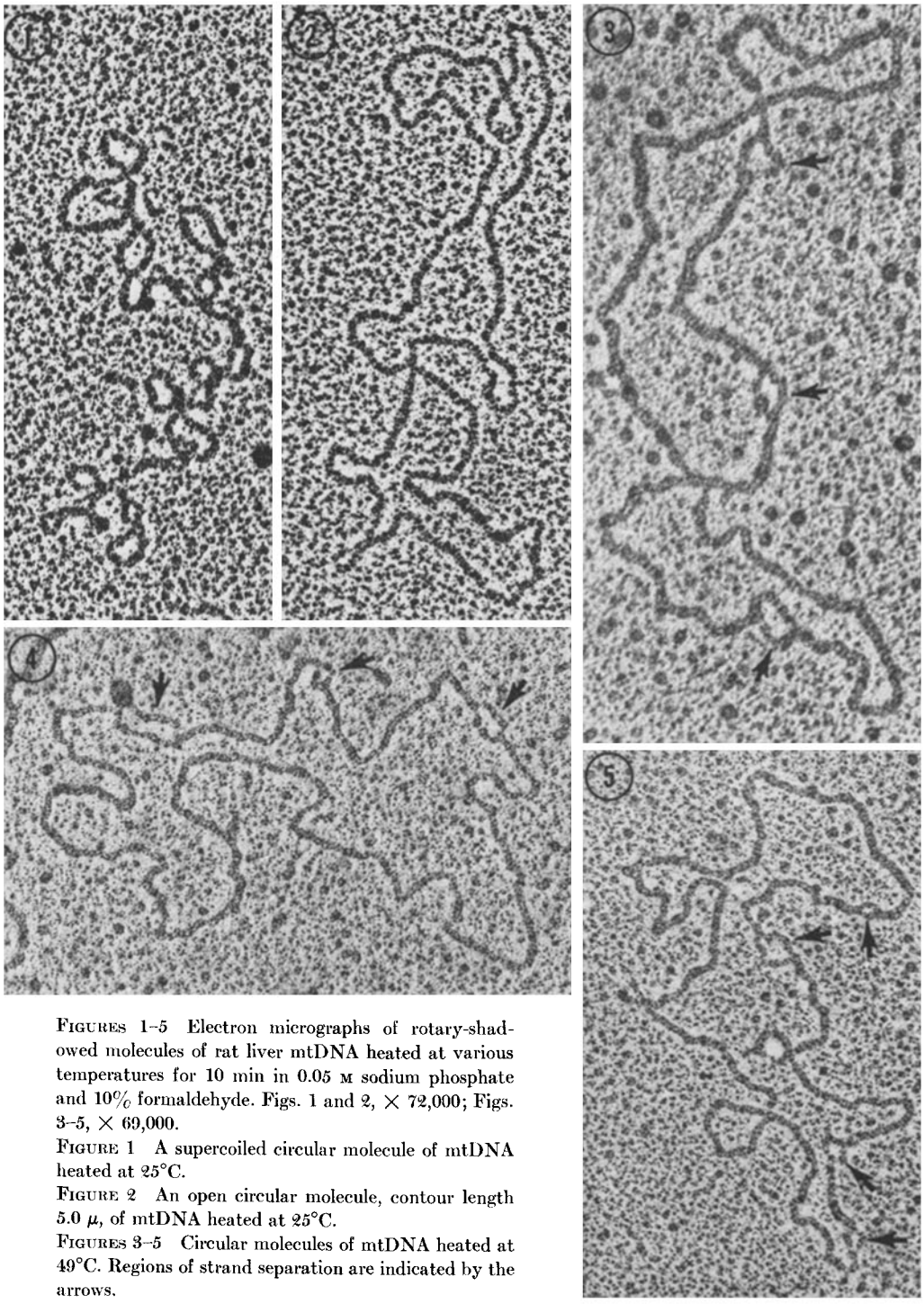
Random numbers were taken from Fisher and Yates (1963). Probabilities (*P*) mentioned in the text resulted from analyses of variance.

RESULTS

It was determined by electron microscope examination that at least 95% of each of the mtDNAs used in the present experiments was circular. Each of the mtDNAs comprised highly twisted or supercoiled circular molecules (Fig. 1) as well as open circular molecules (Fig. 2) as reported before for rat liver mtDNA (Kirschner et al., 1968) and mtDNAs of other metazoan animals (see reviews of Swift and Wolstenholme, 1969; Wolstenholme et al., 1971). The supercoiled form of mtDNA, referred to as component I, has been shown to be a covalently closed molecule (Kroon et al., 1966; Dawid and Wolstenholme, 1967; Clayton and Vinograd, 1967); that is, all of the phosphodiester bonds in both polynucleotide chains are intact (Vinograd et al., 1965). Open circular molecules (component II) differ in that they contain at least one phosphodiester bond break in one of the strands.

Thermal Melting Curves

Thermal melting curves for rat liver mtDNA and for rat liver nuclear DNA are given in Fig. 6. The presence of formaldehyde resulted in considerable lowering of melting temperatures, as has been found for other DNAs (Grossman et al., 1961; Freifelder and Davison, 1962, 1963). The mtDNA was found to have a T_m value (the temperature at one-half transition [Doty et al., 1959]) similar to that of the nuclear DNA examined under similar conditions. However, the mtDNA containing 51% supercoiled molecules showed an increase in absorbance of about one-half that shown by nuclear DNA. That this reflected resistance to denaturation of the covalently closed molecules was supported by the following: a preparation of mtDNA containing 91% supercoiled molecules showed only about a



FIGURES 1–5 Electron micrographs of rotary-shadowed molecules of rat liver mtDNA heated at various temperatures for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. Figs. 1 and 2, × 72,000; Figs. 3–5, × 69,000.

FIGURE 1 A supercoiled circular molecule of mtDNA heated at 25°C.

FIGURE 2 An open circular molecule, contour length 5.0 μ, of mtDNA heated at 25°C.

FIGURES 3–5 Circular molecules of mtDNA heated at 49°C. Regions of strand separation are indicated by the arrows.

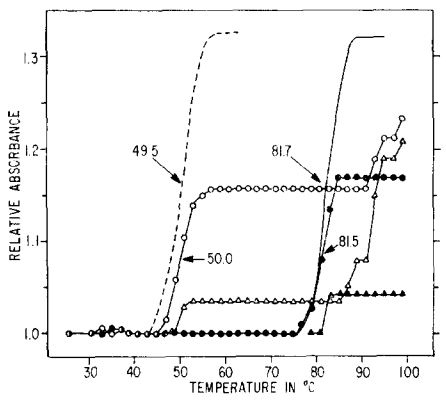


FIGURE 6 The effect on absorbancy at 260 mμ of continuously heating rat liver mtDNA and nuclear DNA. The unbroken line represents nuclear DNA in 0.05 M sodium phosphate, and the dashed line represents nuclear DNA in 0.05 M sodium phosphate and 10% formaldehyde. ●——● and ▲——▲, mtDNA containing 51% and 91% supercoiled molecules, respectively, in 0.05 M sodium phosphate. ○——○ and △——△, mtDNA containing 51% and 91% supercoiled molecules, respectively, in 0.05 M sodium phosphate and 10% formaldehyde. T_m values are indicated for the nuclear DNAs and the mtDNAs containing 51% supercoiled molecules.

10% increase in absorbance compared to nuclear DNA. When both preparations of mtDNA heated in 0.05 M sodium phosphate without formaldehyde were removed from the spectrophotometer at the end of the experiment, rapidly cooled, and examined in the electron microscope, they were found to contain apparently double-stranded supercoiled molecules. At higher temperatures in the presence of formaldehyde, both mtDNAs exhibited a second phase of increase in absorbance. When these DNAs were examined in the electron microscope at the end of the experiment, no supercoiled molecules were found. This indicates that the later increase in absorption resulted from breakage and denaturation of covalently closed circles.

Molecular Configurations of mtDNA at Different Temperatures

The initial phase of absorbance increase in the mtDNA preparations was taken to represent the transition of open circular (component II) molecules from double-stranded to single-stranded forms. For the mtDNA containing 51% supercoiled molecules, the temperature range for this transition in the presence of formaldehyde was about 45°–57°C. Samples of mtDNA from a preparation containing approximately 50% supercoiled molecules were therefore heated in the presence of formaldehyde at 25°, 30°, 35°, 40°, 45°, 47°, 48°, 49°, 50°, 51°, 55°, 60°, 70°, 80°, 90°, and 100°C for 10 min, quenched in ice, and examined in the electron microscope.

mtDNA heated at 25°C contained the same proportion of supercoiled molecules found in the original preparation (Fig. 7). (A supercoiled circle was defined as one characterized by a tight coiling of the molecule into one or more axes [Figs. 1, 8, and 9]. It is usually possible to define at least 10 points of crossover of the strands in these molecules. Some, however, are too tightly coiled to define points of crossover or to measure.) Reduction of the proportion of supercoiled molecules accompanied increase in temperature. mtDNA heated at temperatures of 40°C through 50°C contained only apparently double-stranded open circular forms. In some molecules from preparations heated at 48°, 49°, and 50°C, strand separations were apparent (Figs. 3–5). A consideration of the positions of these strand separations will be made in detail in a later section. In preparations heated at 51°C, loosely supercoiled double-stranded circular molecules were again apparent (Fig. 8). This preparation also contained filaments which were kinky and had less contrast than the supercoiled molecules. Taking into consideration the thermal denaturation data (Fig. 6), and the results of the similar experiments of Inman (1966; 1967) and Follett and Crawford (1967 *a*, *b*; 1968), these filaments are interpreted as representing single polynucleotide strands of DNA. They occurred in three forms: 5% of this DNA consisted of circles with a mean contour length of 3.36 μ (Figs. 10 and 13); 6% consisted of linear pieces with a range of lengths from 1.2 μ to 3.5 μ (mean of the modal lengths = 3.16 μ) (Fig. 13); and 89% consisted of molecules measuring up to 7.3 μ, which could be interpreted as a single-stranded linear piece joined at one or a small number of points to a single-stranded circle (Fig. 11), and produced by incomplete strand separation of a native component II molecule. The single-stranded forms were found to represent approximately 50% of the total DNA and were therefore interpreted as representing the product of denaturation of only component II double-stranded molecules. This was

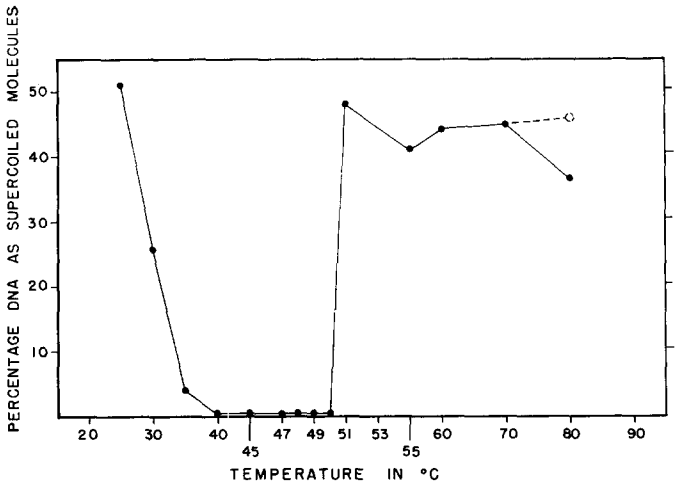


FIGURE 7 Percentage of mtDNA as supercoiled circular molecules in samples of a preparation heated at various temperatures for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. Up to 80°C, most of the single-strands of DNA were intact and it was therefore possible to make accurate measurements of the amount of DNA in any one form. After heating at 90°C, supercoiled molecules were present but much of the single-stranded DNA was fragmented or clumped making quantitative estimates unreliable. Supercoiled molecules were not found after heating at 100°C. The dotted ring at 80°C represents the sum of double-stranded molecules which showed regions of strand separations and supercoiled molecules.

confirmed by similarly treating a mtDNA preparation originally containing 91% supercoiled molecules. In this case, 14% of the DNA was found to be single-stranded, and the remainder appeared as supercoils.

In most of the forms comprising a single-stranded circle and a single-stranded linear piece, the length of the linear piece was about equal to the length of the circle (Fig. 14). This indicates that there was only one phosphodiester bond break in one of the polynucleotide strands of the native circle. The positions of the connections (when there was more than one) on the molecules seemed to be random relative to each other. Also, such connections were still apparent between 47% of the single-stranded DNA in samples heated to 80°C.

Separate single-stranded circular and linear mtDNA molecules were also found in samples heated at temperatures up to and including 90°C. In mtDNA heated to 100°C, single-stranded DNA was seen only as short linear pieces.

FIGURES 8–12 Electron micrographs of rotary-shadowed circular molecules of rat liver mtDNA heated at various temperatures for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. All micrographs, × 74,000.

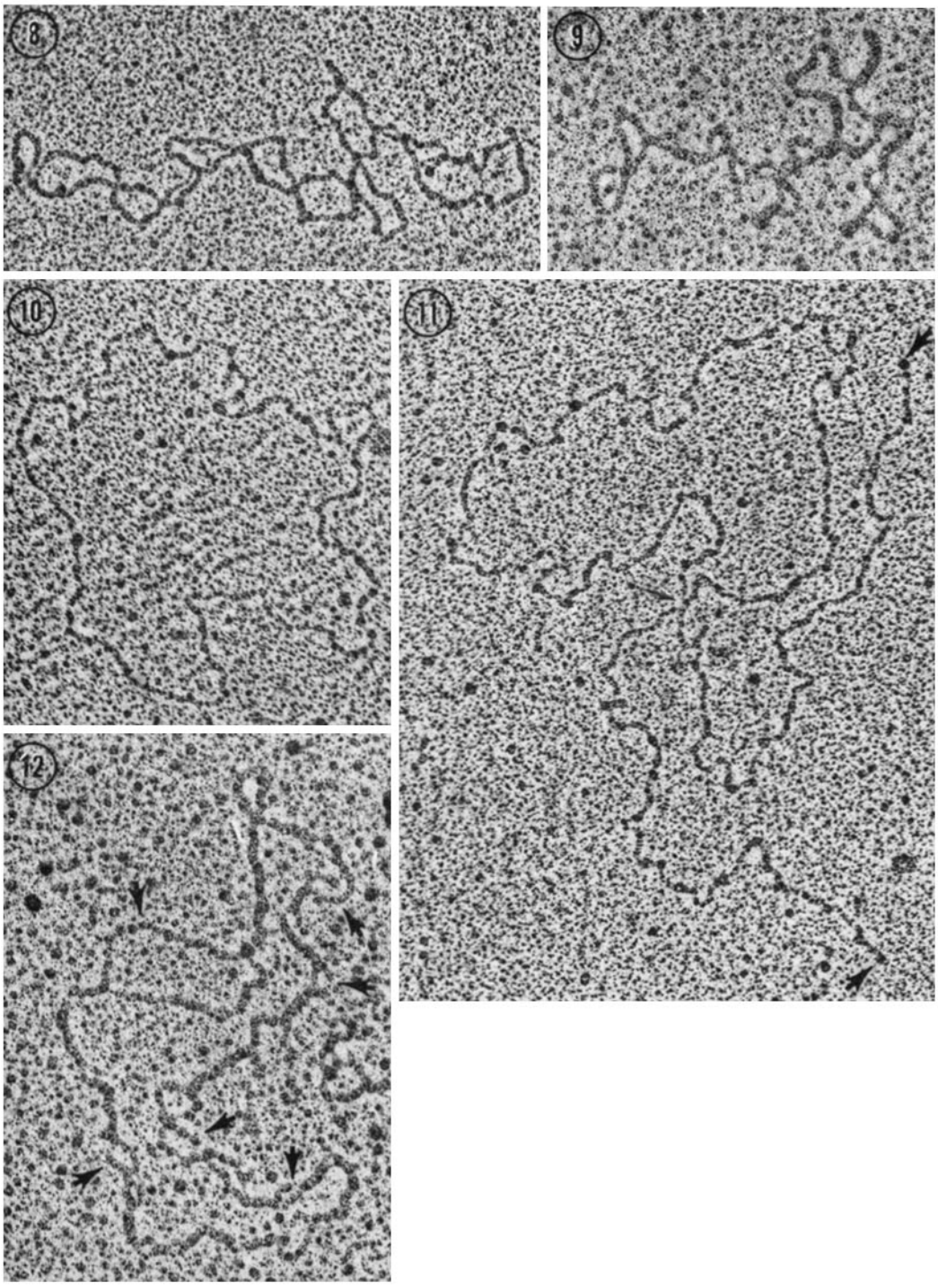
FIGURE 8 Supercoiled molecule of mtDNA heated at 51°C.

FIGURE 9 Supercoiled molecule of mtDNA heated at 80°C.

FIGURE 10 A single-stranded molecule of mtDNA heated at 51°C. Contour length 3.38 μ.

FIGURE 11 A molecule of mtDNA heated at 51°C which can be interpreted as comprising a single-stranded circle (contour length 3.30 μ) and a single-stranded linear filament (length 3.10 μ) between which there is one point of contact (small arrow). The large arrows indicate the ends of the linear filament.

FIGURE 12 A molecule of mtDNA heated at 80°C in which strand separations are visible (some of which are indicated by arrows) in the apparently double-stranded molecule.


8
9
10
11
12

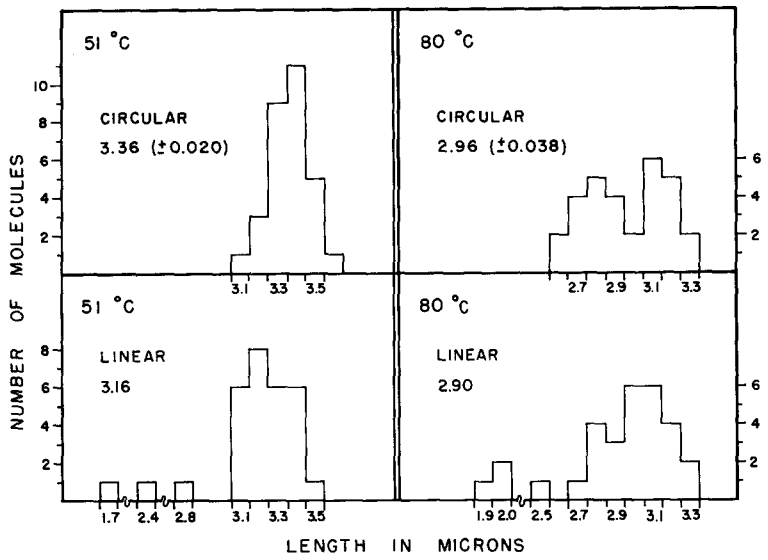


FIGURE 13 Frequency distributions of the lengths of single-stranded circular and linear molecules in rat liver mtDNA heated at 51°C and 80°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. The means and standard errors of the circular molecules and the means of the linear molecules are given.

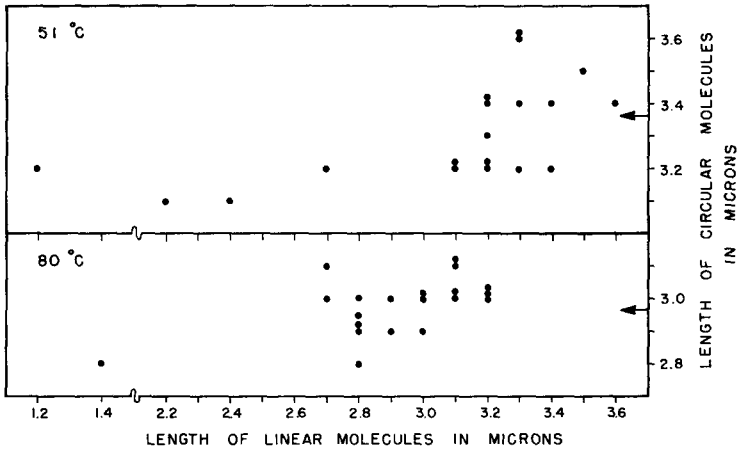


FIGURE 14 Plots of the lengths of connected circular and linear single-stranded rat liver mtDNA molecules from samples heated at 51°C and at 80°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. The arrows on the right ordinate indicate the positions of the mean lengths of single circular molecules from the respective preparations.

Supercoiled molecules were also found in all of the samples heated up to and including 90°C (Figs. 7 and 9). They accounted for between 41% and 45% of the DNA in the 55°, 60°, and 70°C samples. The supercoiling appeared much tighter at the higher temperatures (cf. Figs. 8 and 9).

In the sample heated at 80°C, an apparently double-stranded open circular form was present which showed up to 15 regions of strand separation (Fig. 12). An attempt was made to compare the positions of the strand separations of molecules showing the same number of separations. No clear groupings were found. The most likely explanation of these forms is that they resulted from a single phosphodiester bond break occurring in a covalently closed molecule in which the strands were extensively cross-linked by formaldehyde. The following observations support this interpre-

tation. The sum of the percentage of DNA in this form and in the supercoiled form in the sample heated at 80°C was similar to the per cent of DNA in the supercoiled form in the sample heated at 70°C (Fig. 7). mtDNA heated at 90° and 100°C included these forms and also apparently double-stranded linear DNA molecules showing regions of strand separation. The latter molecules could result if a covalently closed molecule first became cross-linked then broke at positions close together on the two strands. The breakdown and partial denaturation of covalently closed molecules at high temperatures in the presence of formaldehyde is also consistent with the finding of a second phase of absorbance increase of mtDNA at high temperatures under these conditions (Fig. 6).

Denaturation Mapping

Of the molecules heated at 48°C, 5% were found to have a single strand separation. Of the molecules heated at 49°C, 13% had a single strand separation, 4% had two strand separations, 7% had three strand separations (which together average 4.8% the length of a molecule), and 2% had four strand separations. The remaining molecules did not have detectable regions of strand separation. Molecules showing strand separations were assumed to be component II.

Molecules having three regions of strand separation were first compared. In a circular molecule, unlike the lambda phage molecules studied by Inman (1966, 1967), there is no beginning or natural reference point. Also, there is no indication of direction. For the purpose of comparison, the following procedure was therefore adopted. Each circular molecule was converted to a linear rod. The lengths of native and denatured regions of each molecule were standardized by converting them to percentage lengths of the molecule. The three regions of strand separation were always contained within a segment of the molecule equal to less than one-half the length of the molecule. The midpoint of this segment in each molecule was taken as the common point by which the molecules were lined up. The larger of the two regions of native DNA between the three strand separations was placed to the left in each molecule, thus defining direction. 25 such arrangements of molecules and the result of comparing them are shown in Fig. 15. There is a distinct indication of three common regions of strand separation in the molecules compared. Also, as

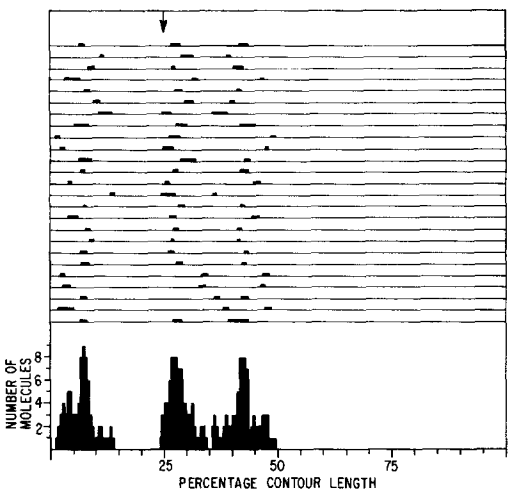


FIGURE 15 Denaturation maps of 25 rat liver mtDNA molecules each containing three regions of strand separation, obtained by heating at 49°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. For the purpose of comparison, each circular molecule was converted to a linear rod. The lengths of the regions of strand separation (thick lines) and the native regions (thin lines) were standardized by converting them to percentage lengths of the molecule. The three regions of strand separation were always contained within a segment of the molecule equal to less than half the length of the molecule. The midpoint of this segment in each molecule was taken as the common point by which the molecules were lined up (arrow). The larger of the two regions of native DNA between the three strand separations were placed to the left in each molecule, thus defining direction. The histogram at the bottom of the diagram has been constructed from the 25 maps to show the variation in occurrence of the regions of strand separation at intervals of 0.5% (about 250 A) around the molecule.

mentioned above, the regions of strand separation, which are assumed to be adenine-thymine–rich regions, are limited to less than 50% of the molecule.

mtDNA molecules with two regions of strand separation were compared with the three strand separation map. First, the mean positions of the three groups of the histogram in Fig. 15 were calculated and marked by arrows on a map as reference points (Fig. 16). Each molecule containing two regions of strand separation was then converted to a linear rod, and the lengths of native and denatured regions were converted to percentage lengths of the molecule as before. The smaller strand separation was placed to the

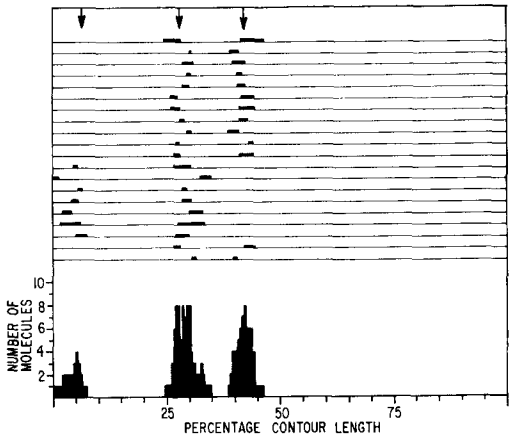


FIGURE 16 Denaturation maps of 20 rat liver mtDNA molecules each containing two regions of strand separation, obtained by heating at 49°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. The positions of the strand separations are compared to the mean positions of the three groups shown in Fig. 15 and indicated by arrows at the top of this diagram. Each molecule was converted to a linear rod. The lengths of the regions of strand separation (thick lines) and the native regions (thin lines) were standardized by converting them to percentage lengths of the molecule. The smaller strand separation was placed to the left and the midpoint of the smaller segment of the molecule containing the two strand separations was lined up with a point equidistant from two of the arrows. The two arrows chosen for an individual molecule were those whose distance apart was most similar to the distance between the two strand separations. The histogram at the bottom of the diagram has been constructed to show the variation in occurrence of the regions of strand separation at intervals of 0.5% (about 250 A) around the molecule.

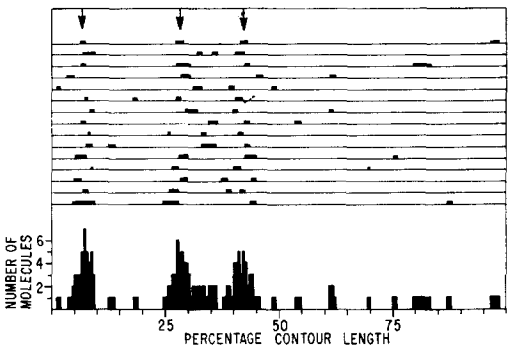


FIGURE 17 Denaturation maps of 15 rat liver mtDNA molecules each containing four regions of strand sepration obtained by heating at 49°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. The positions of the strand separations are compared to the mean position of the three groups shown in Fig. 15 and indicated by arrows at the top of this diagram. Each molecule was converted to a linear rod. The lengths of the regions of strand separation (thick lines) and the native regions (thin lines) were standardized by converting them to percentage lengths of the molecule. For each molecule, the midpoint of a segment containing either three or all four strand separations was lined up with the midpoint between the two outside arrows. The number of single-stranded regions included in this segment and their orientation was chosen to give maximum overlap. The histogram at the bottom of the diagram has been constructed to show the variation in occurrence of the regions of strand separation at intervals of 0.5% (about 250 A) around the molecule.

left and the midpoint of the smaller segment of the molecule containing the two strand separations was lined up with a point equidistant from two of the arrows. The two arrows chosen for an individual molecule were those whose distance apart was most similar to the distance between the two strand separations. The result of comparing these molecules (Fig. 16) is again in agreement with there being three common regions of strand separation.

mtDNA molecules with four regions of strand separation were next compared to the three strand separation map (Fig. 17) in a similar way to that used for molecules with two strand separations. In this case, however, the midpoint of a segment of a molecule containing either three or four strand separations was lined up with the midpoint between the two outside arrows. The number of regions of strand separation included in this segment and their orientation was chosen to give maximum overlap. When these molecules were compared (Fig. 17), again three common regions could be discerned, but the fourth mapped more or less at random. This latter observation may be an indication of the limitation of the technique for this DNA.

In order to test whether the three common groupings resulting from comparing molecules containing three strand separations (Fig. 15) are likely to be generated by random denaturation within one-half of the molecule, a test was made using artificial molecules containing three strand separations, the positions and sizes of which were generated by random numbers. One set of random numbers was used to determine the mean position of three regions of strand separation within the equivalent of 48.5% of the mole-

cule length. A second set of random numbers was used to determine the size of each separation equivalent to the range of separation found in mtDNA molecules; 0.5–4.5% of the map length. Exactly the same procedure as that employed to compare the mtDNA molecules showing three strand separations was then used to compare the positions of strand separation generated by random numbers. This was done for each of two sets of 25 molecules. In each case, the resultant histograms showed only two groups: a relatively narrow group to the left and a broad group to the right. The group to the left in each histogram had a significantly higher mean position (P = <0.001 in each case) on the map and a significantly higher variance (P = <0.001 and $P \simeq$ 0.025) than the left hand group in Fig. 15. These results are, therefore, in agreement with the interpretation that the three regions of the rat liver mtDNA molecule which melt at a lower temperature are located at specific positions on the molecule.

Molecular Lengths of MtDNA at Different Temperatures

Contour lengths of the different molecular forms in samples heated to different temperatures are summarized in Fig. 18. Supercoiled molecules at 25°C had about the same length as open circular molecules. The presence of formaldehyde at 25°C did not substantially alter contour lengths. Up to 49°C, the mean contour length of the double-stranded open forms (which must include

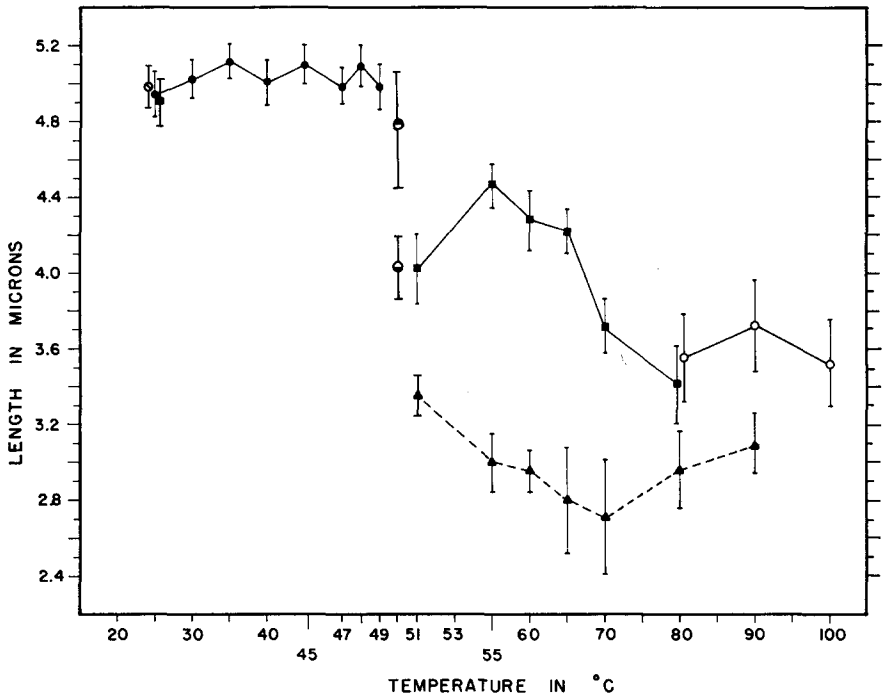


FIGURE 18 The mean contour lengths and standard deviations of circular molecules in samples of a preparation of rat liver mtDNA heated at various temperatures for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. Each point represents the mean of 30 molecules. —●—, double-stranded open molecules. —■—, double-stranded supercoiled molecules. —○—, double-stranded molecules showing strand separations. —▲—, single-stranded molecules. The two points at 50°C represent open circular molecules showing no strand separation (◓) and open circular molecules showing either five, six, or seven strand separations (◒). In the 25°, 65°, 70°, and 80°C samples, many of the supercoiled molecules were too compact to measure accurately. The values given for supercoiled molecules in these samples are, therefore, representative only of the less compact supercoiled molecules. The single point, ⊘, represents the mean of 30 open circular molecules from a sample stored in 0.05 M sodium phosphate but not exposed to 10% formaldehyde.

covalently closed molecules in the samples heated at 40°–49°C) showed little variation about a value of 5.0 μ. Of the open circular molecules measured from the 50°C sample, those having no strand separations showed a slight drop in mean contour length (to 4.73 μ) but the molecules showing either five, six, or seven strand separations were found to have decreased in mean contour length to 4.02 μ.

The mean lengths of the double-stranded supercoiled molecules found in samples heated at 51°C and above decreased with increase in temperature, and were less than those of the double-stranded molecules found in samples heated at lower temperatures. The mean length of the open double-stranded forms showing strand separations in the 80°C sample was similar to that of the supercoiled molecules and showed very little variation with increase in temperature. Single-stranded circular molecules were shorter than the supercoiled forms in each sample, and from 51°C through 70°C showed a continuous decrease in length to a value of only 55% the mean length of native open circular molecules.

DISCUSSION

The results of denaturation mapping of molecules heated at 49°C indicate that in at least 25% of the mtDNA molecules, one-half of the molecule is distinguished from the other half by regions which denature at lower temperatures. There appear to be at least three such regions. Under the conditions of our experiments, Follett and Crawford (1968) found that the first regions in which strand separations occur are rich in adenine and thymine. It has been shown by the use of ultracentrifuge and chromatography techniques (Hogness and Simmons, 1964) as well as denaturation mapping (Inman, 1966; 1967) that one-half of the lambda phage DNA molecule differs distinctly in average base composition from the other half. Distinct regions showing differential melting were demonstrated for lambda phage DNA over a range of 3°C (Inman, 1966) and 4°C (Inman, 1967), for P2 bacteriophage DNA over a range of 7°C (Inman and Bertani, 1969), for human papilloma virus DNA over a range of 8°C (Follett and Crawford, 1967 *b*), and for polyoma virus DNA at two temperatures, 1°C apart (Follett and Crawford, 1968). As the early melting regions in mtDNA can only be detected at the very beginning of strand separation and are very short, they probably represent regions with adenine-thymine contents which differ from the rest of the molecule to a lesser degree than do the early melting regions of the virus DNAs which have been examined.

There is no indication at the present time of a possible functional significance for the three early melting regions of the rat liver mtDNA molecule.

The present mapping results are consistent with the interpretation that mtDNA molecules comprise only one species with respect to their base sequences. However, particularly as it was possible to successfuly compare molecules showing only a small amount of strand separation, and as these accounted for no more than 25% of the component II molecules in the sample, other interpretations are not ruled out.

By 51°C, the component II molecules separated into a single-stranded circle and a single-stranded linear piece of similar length, as indicated by previous sedimentation and electron microscope studies (Dawid and Wolstenholme, 1967; Borst et al., 1967; Nass, 1969). Separation was either total, or the single strands remained connected at one or a small number of points. As these points of attachment (when there was more than one) appeared to be random relative to each other, and as such connections were still apparent between single-stranded DNA molecules heated at 80°C, they most probably resulted from incomplete separation of the strands due to cross-linking by formaldehyde (Freifelder and Davison, 1963) rather than to specific hydrogen bonding. Similar molecular forms were found when open circular polyoma virus DNA was heated to 70°C (Bourguignon, 1968).

The shorter lengths of single-stranded DNA molecules produced by heating in formaldehyde have also been noted by Follett and Crawford (1967 *b*) for human papilloma virus DNA molecules. This contracted secondary structure of single-stranded mtDNA seems likely to account for the shorter length of partially denatured molecules in the 50°C sample and also the shorter length of the supercoiled form found in samples heated at 51°C and above. Inman and Bertani (1969) reported that progressive shortening of P2 bacteriophage DNA molecules accompanied increased strand separation.

The present data are in agreement with previous work indicating the resistance of covalently closed circular DNA molecules to denaturation

(Vinograd and Lebowitz, 1966; Dawid and Wolstenholme, 1967; Borst et al., 1967; Follett and Crawford 1967 *a*, 1968; Nass, 1969; Renger and Wolstenholme, 1970; Leffler et al., 1970).

With increasing temperature, in the presence of formaldehyde, supercoiled covalently closed circular mtDNA molecules (component I) first convert to an open circle (component I′) and then at the temperature at which component II molecules are completely denatured, to a second supercoiled form (component I″). Similar observations have been made concerning component I of human papilloma virus DNA (Follett and Crawford, 1967 *a*) and polyoma virus DNA (Follett and Crawford, 1968). Following the interpretation of Follett and Crawford, the observations for mtDNA may be explained by assuming that heating the covalently closed molecule results in the separation of a small number of base pairs which allow the superhelical forms to unwind. This results in a simple open circular form, I′, in which both strands are intact. Further heating causes separation of more base pairs, which forces superhelical turns of opposite sense to those of component I into the molecule. The change in sense of the superhelical turns has been argued from the results of experiments with the dye ethidium bromide, which intercalates between the bases of the DNA molecule (Waring, 1965). Intercalation of increasing amounts of the dye into covalently closed DNA molecules unwinds the helix and the molecule converts first to an open circle (I′) and then to a second supercoiled form (I″) (Crawford and Waring, 1967 *a*, *b*; Ruttenberg et al., 1968).

The present electron microscope observations are in agreement with the results of previous sedimentation studies concerning covalently closed molecules of mtDNA (Dawid and Wolstenholme, 1967; Borst et al., 1967). Exposure to increasing pH or increasing temperature in the presence of formaldehyde, resulted first in a lowering of the sedimentation coefficient of component I followed by a steady increase to a value indicating a very compact structure. This was interpreted as resulting from progressive unwinding of the DNA double helix with increased degrees of hydrogen bond breakage between the base pairs. The final component (I^{alk}) was interpreted as representing two circular single-stranded molecules, topologically interlocked due to their covalent closure, but completely denatured and collapsed. In the present study, confirmation of the structure of this final product of denaturation of component I was prevented due to extensive cross-linking of these molecules by formaldehyde at higher temperatures, presumably resulting in partial retention of the double helical form.

We wish to thank Janice A. Hunter for printing and measuring molecules. We are indebted to Drs. Hewson Swift and Jerry E. Manning for valuable suggestions during the course of this work and preparation of the manuscript.

This study was supported by United States Public Health Service Research Grants No. GM 18375, HD-174, HE 04442, and GM 00093, and American Cancer Society Grant No. E-531.

Received for publication 22 October 1971, and in revised form 14 January 1972.

REFERENCES

BORST, P., G. J. C. M. RUTTENBERG, and A. M. KROON. 1967. Mitochondrial DNA. I. Preparation and properties of mitochondrial DNA from chick liver. *Biochim. Biophys. Acta.* **149**:140.

BORST, P., E. F. J. VAN BRUGGEN, C. J. C. M. RUTTENBERG, and A. M. KROON. 1967. Mitochondrial DNA. II. Sedimentation analysis and electron microscopy of mitochondrial DNA from chick liver. *Biochim. Biophys. Acta.* **149**:156.

BOURGUIGNON, M-F. 1968. A denaturation map of polyoma virus DNA. *Biochim. Biophys. Acta.* **166**: 242.

CLAYTON, D. A., and J. VINOGRAD. 1967. Circular dimer and catenate forms of mitochondrial DNA in human leukaemic leucocytes. *Nature (London).* **216**:652.

CRAWFORD, L. V., and M. J. WARING. 1967 *a*. Supercoiling of polyoma virus DNA measured by intercalation with ethidium bromide. *J. Mol. Biol.* **25**:23.

CRAWFORD, L. V., and M. J. WARING. 1967 *b*. The supercoiling of papilloma virus DNA. *J. Gen. Virol.* **1**:387.

DAWID, I. B., and D. R. WOLSTENHOLME. 1967. Ultracentrifuge and electron microscope studies on the structure of mitochondrial DNA. *J. Mol. Biol.* **28**:233.

DOTY, P., H. BOEDTKER, J. R. FRESKO, R. HASELKORN, and M. LITT. 1959. Secondary structure of ribonucleic acids. *Proc. Nat. Acad. Sci. U. S. A.* **45**:482.

FISHER, R. A., and F. YATES. 1963. Statistical Tables for Biological, Agricultural and Medical Research. Oliver and Boyd Ltd., Edinburgh.

FOLLETT, E. A. C., and L. V. CRAWFORD. 1967 *a*.

Electron microscope study of the denaturation of human papilloma virus DNA. I. Loss and reversal of supercoiling turns. *J. Mol. Biol.* **28**:455.

FOLLETT, E. A. C., and L. V. CRAWFORD. 1967 *b*. Electron microscope study of the denaturation of human papilloma virus DNA. II. The specific location of denatured regions. *J. Mol. Biol.* **28**:461.

FOLLETT, E. A. C., and L V. CRAWFORD. 1968. Electron microscope study of the denaturation of polyoma virus DNA. *J. Mol. Biol.* **34**:565.

FREIFELDER, D., and P. F. DAVISON. 1962. Hyperchromicity and strand separation in bacterial DNA. *Biophys. J.* **2**:249.

FREIFELDER, D., and P. F. DAVISON. 1963. Physiochemical studies on the reaction between formaldehyde and DNA. *Biophys. J.* **3**:49.

FREIFELDER, D., and A. K. KLEINSCHMIDT. 1965. Single strand breaks in duplex DNA of coliphage T7 as demonstrated by electron microscopy. *J. Mol. Biol.* **14**:271.

GROSSMAN, L., S. S. LEVINE, and W. S. ALLISON. 1961. The reaction of formaldehyde with nucleotides and T2 bacteriophage DNA. *J. Mol. Biol.* **3**:47.

HOGNESS, D. S., and J. R. SIMMONS. 1964. Breakage of λdg DNA: chemical and genetic characterization of each isolated half-molecule. *J. Mol. Biol.* **9**:411.

INMAN, R. B. 1966. A denaturation map of the λ phage DNA molecule determined by electron microscopy. *J. Mol. Biol.* **18**:464.

INMAN, R. B. 1967. Denaturation maps of the left and right sides of the lambda DNA molecule determined by electron microscopy. *J. Mol. Biol.* **28**:103.

INMAN, R. B., and G. BERTANI. 1969. Heat denaturation of P2 bacteriophage DNA: compositional heterogeneity. *J. Mol. Biol.* **44**:533.

KIRSCHNER, R. H., D. R. WOLSTENHOLME, and N. J. GROSS. 1968. Replicating molecules of circular mitochondrial DNA. *Proc. Nat. Acad. Sci. U. S. A.* **60**:1466.

KROON, A. M., P. BORST, E. F. J. VAN BRUGGEN, and G. J. C. M. RUTTENBERG. 1966. Mitochondrial DNA from sheep heart. *Proc. Nat. Acad. Sci. U. S. A.* **56**:1836.

LEFFLER, A. T., II., E. CRESKOFF, S. W. LUBORSKY, V. MCFARLAND, and P. T. MORA. 1970. Isolation and characterization of rat liver mitochondrial DNA. *J. Mol. Biol.* **48**:455.

MANDELL, J. D., and A. D. HERSHEY. 1960. A fractionating column for analysis of nucleic acids. *Anal. Biochem.* **1**:66.

NASS, M. M. K. 1969. Mitochondrial DNA. II. Structure and physiochemical properties of isolated DNA. *J. Mol. Biol.* **42**:529.

RABINOWITZ, M., J. SINCLAIR, L. DESALLE, R. HASELKORN, and H. H. SWIFT. 1965. Isolation of deoxyribonucleic acid from mitochondria of chick embryo heart and liver. *Proc. Nat. Acad. Sci. U. S. A.* **53**:1126.

RENGER, H. C., and D. R. WOLSTENHOLME. 1970. Kinetoplast deoxyribonucleic acid of the hemoflagellate *Trypanosoma lewisi*. *J. Cell Biol.* **47**:689.

RUTTENBERG, G. J. C. M., E. M. SMITH, P. BORST, and E. F. J. VAN BRUGGEN. 1968. The number of superhelical turns in mitochondrial DNA. *Biochim. Biophys. Acta.* **157**:429.

SWIFT, H., and D. R. WOLSTENHOLME. 1969. Mitochondria and chloroplasts: nucleic acids and the problem of biogenesis. *In* Handbook of Molecular Cytology. A. Lima de Faria, editor. North Holland Publishing Co., Amsterdam. 972–1046.

VINOGRAD, J., and J. LEBOWITZ. 1966. Physical and topological properties of circular DNA. *J. Gen. Physiol.* **49**:103.

VINOGRAD, J., J. LEBOWITZ, R. RADLOFF, R. WATSON, and P. LAIPIS. 1965. The twisted circular form of polyoma viral DNA. *Proc. Nat. Acad. Sci. U. S. A.* **53**:1104.

WARING, M. J. 1965. Complex formation between ethidium bromide and nucleic acids. *J. Mol. Biol.* **13**:269.

WOLSTENHOLME, D. R., K. KOIKE, and H. C. RENGER. 1971. Form and structure of mitochondrial DNA. *In* Oncology 1970. A. Cellular and molecular mechanisms of carcinogenesis. B. Regulation of gene expression. R. L. Clark, R. W. Cumley, J. E. McCoy, and M. M. Copeland, editors. Year Book Medical Publishers, Inc., Chicago. **1**:627–648.