HYPERCHROMICITY AND STRAND SEPARATION IN BACTERIAL DNA

DAVID FREIFELDER *and* PETER F. DAVISON
From the Department of Biology, Massachusetts Institute of Technology, Cambridge

ABSTRACT Studies of the per cent of strand separation of N^{14}-N^{15} hybrid *coli* DNA heated to various temperatures in formaldehyde have shown that the process of strand separation is a function of temperature and formaldehyde concentration and is directly related to the measured hyperchromicity. No strands separate until about 75 per cent of full hyperchromicity is obtained, and even at apparently full hyperchromicity a large fraction of the strands may be held together, possibly by guanine-cytosine–rich regions.

INTRODUCTION

The heat denaturation of DNA solutions with the concomitant increase of optical density (hyperchromicity) has been identified with the breakdown of the hydrogen bonds linking the complementary base pairs, followed by the separation of the polynucleotide strands (1). A graph relating optical density with temperature is known as a "melting-out curve"; both the midpoint of this curve and the mean density of any given DNA have been correlated with the mean base composition of the DNA (2–4).

The breadth of the melting-out curve—*i.e.*, the temperature range between the commencement and the completion of denaturation—is less in the case of the synthetic adenine-thymine polymer than for any DNA preparation studied (2). In view of the correlation between melting-out temperature and base composition, and since the bases are not randomly distributed (5, 6), it seems probable that the breadth of the melting-out diagram results from variations in the base distributions along the DNA molecules. However, it is not clear to what degree the melting-out of a DNA molecule is a cooperative phenomenon: whether, on the one hand, the separation of the strands of the double helix at any one point in the molecule leads immediately to the complete denaturation of that molecule, or whether, on the other hand, each short sequence of bases along a molecule melts out at a temperature determined by the local base composition. In these two situations the width of the melting-out curve would reflect either the "melting" range of different molecules in the polydisperse solution (intermolecular heterogeneity) or the successive melt-

ing of different regions of individual molecules (intramolecular heterogeneity). There is some evidence at present that the latter process contributes substantially to the breadth of the melting-out curve. For example T2 and T7 bacteriophage DNA, which are physically homogeneous (7, 8), melt out across a temperature range not much narrower than that of bacterial and animal DNA preparations (Fig. 1), and Beer and Thomas (9) have obtained electron micrographs which appear to show single T2 DNA molecules partly native and partly denatured.

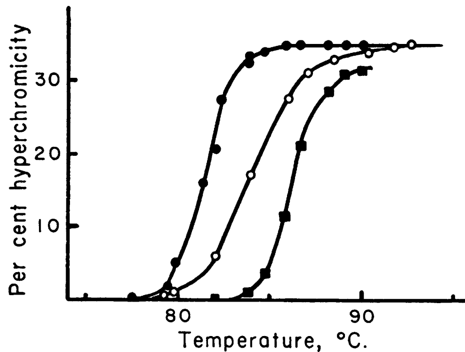

FIGURE 1 Melting-out curves of trout sperm (○), T2 (●), and T7 (■) bacteriophage DNA preparations in 0.15 M sodium chloride, 0.015 M sodium citrate.

Furthermore, although substantial regions of the melting-out curves of several pairs of bacterial DNA's overlap (*e.g., pneumococcus* and *serratia* (2)), each DNA has a distinct mean density, and the bands in cesium chloride show little if any overlap (3). This implies that there exist no molecules with the density corresponding to the extremes of the melting range. The studies by Geiduschek (10) on the reversibility of thermal denaturation of native DNA may be similarly interpreted if it is accepted that reversibility depends upon the persistence in a molecule of at least one undissociated region which serves to maintain the registration of the strands. Until the onset of irreversibility, at 50 to 60 per cent of full hyperchromicity, the increase in absorbance must reflect the denaturation of substantial regions within many of the molecules.

The above experiments all suggest that much of the breadth of the thermal denaturation curve results from intramolecular heterogeneity. We have studied the denaturation process by a method complementary to that of Geiduschek, *i.e.,* by detecting the progressive appearance of N^{14} and N^{15} single strands from a "hybrid" N^{14}-N^{15} DNA preparation (11) under conditions where no base pair hydrogen bonds reform on cooling, *i.e.,* in formaldehyde (HCHO) solutions. Under these conditions reaggregation does not conceal the onset of the separation of the subunits.

EXPERIMENTAL TECHNIQUES

1. *Growth of N^{14}-N^{15} Bacteria and Isolation of the Hybrid DNA.* *E. coli* B containing N^{14}-N^{15} hybrid DNA were grown by a modification of the method of Mesel-

son and Stahl (11). Bacteria grown up in N^{15} synthetic medium were diluted with a rich N^{14} medium; when the titer had doubled (10^9/ml), growth was stopped with iced 0.01 M sodium azide. The bacteria were immediately concentrated to 5 x 10^{10}/ml in 0.01 M sodium benzoate, 0.01 M versene, pH 7, and heated for 20 minutes at 70°C to inactivate enzymes. This procedure was intended to minimize single strand breaks. DNA was isolated by the phenol technique (12), followed by digestion with boiled ribonuclease and repeated ethanol precipitation.

One sample of DNA was prepared by the sodium dodecyl sulfate method of Meselson and Stahl (11).

2. *Denaturation.* The denaturing solution was 0.01 M phosphate, pH 7.8, containing 1, 2, or 4 per cent freshly neutralized (pH 7 – 8.5) HCHO. 0.02 ml DNA (80 μg/ml) was added to 0.2 ml denaturing solution and heated immediately in 2 ml sealed ampoules for 10 minutes (storage of DNA in HCHO at neutral pH causes cross-linking which prevents the separation of the strands). Constancy of heating time is important since, in the presence of HCHO, the hyperchromicity for a given temperature is time-dependent (8, 13). Optical density was measured before and after denaturation against appropriate HCHO solutions.

3. *CsCl Density Gradient Centrifugation.* The banding solution consisted of 0.787 gm recrystallized CsCl and 0.584 gm denaturing solution containing 2 to 4 μg bacterial DNA and 2 μg phage T2 DNA used as a density marker. All runs were at 44,770 RPM and 25°C in a Spinco Model E ultracentrifuge with ultraviolet absorption optics.

The photographs were scanned with a Joyce-Loebl recording microdensitometer. Relative concentrations of single- and double-stranded material were determined from the areas under the peaks, correcting for the change in extinction coefficient occurring on denaturation.

RESULTS AND DISCUSSION

Optical density measurements showed that in the presence of formaldehyde (1 to 4 per cent) the hyperchromicity attained after any period of heating and measured at the ambient temperature was not diminished on cooling or on the addition of CsCl to the concentration required for banding. It was therefore concluded that no hydrogen bonds reform on cooling and hence that no aggregation occurs.

A more critical test of the absence of aggregation was made by separately denaturing in HCHO, N^{14}-N^{14} and N^{15}-N^{15} *coli* DNA preparations. When mixed and banded in CsCl containing HCHO, each DNA banded at the expected density level. In the absence of HCHO, aggregation was clearly manifest by the appearance of DNA banding in a range of densities between the N^{14} and N^{15} levels.

From these studies we conclude that no aggregation of separated strands occurs after heating in HCHO, and therefore that the percentage of material which the banding experiments showed to be dissociated corresponded to that fraction of strands which had separated at the ambient temperature at the end of the heating period.

Typical densitometer traces from these banding experiments are shown in Figure 2. The DNA preparation used for the experiments included in this paper consisted

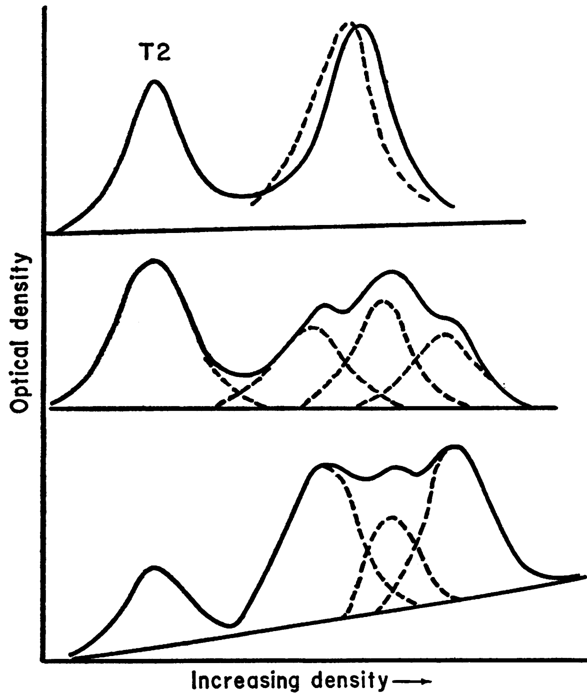

FIGURE 2 Densitometer traces of banded hybrid *coli* DNA at 30, 87, and 100 per cent of full hyperchromicity. The dashed curve in the first diagram indicates the banding position of the undenatured DNA. The dashed curves in the lower diagrams indicate the approximate areas assigned to the N^{14}, hybrid, and N^{15} bands. T2 DNA is the marker in each diagram.

solely of hybrid N^{14}-N^{15} DNA, with no detectable "heavy" (N^{15}-N^{15}) or "light" (N^{14}-N^{14}) material.

With denaturation the density of the partially dissociated hybrid DNA increased; when strand separation occurred, the N^{14} and N^{15} denatured strands moved to the levels in the cell dictated by their density. This density depended upon the concentration of formaldehyde in the CsCl solutions, as shown in Table I.

TABLE I

Formaldehyde concentration in banding solution	Increase in density on denaturation
per cent	*gm/ml*
0	0.015
1	0.012 ± 0.0005
4	0.0045 ± 0.0005

Since the bands of N^{14}, hybrid, and N^{15} DNA, though obvious on inspecting the negatives, were not readily resolved on the densitometer trace, the percentage of strand separation was estimated from the areas of three approximately Gaussian curves drawn to equal the area under the trace. In view of the errors implicit in these measurements large estimated error limits are included in Figs. 3 and 4.

Similar experiments yielding identical results were made on two other hybrid DNA preparations which contained small amounts of heavy (N^{15}-N^{15}) or light (N^{14}-N^{14}) DNA, but in these instances the estimates of strand separation were more difficult to make.

Fig. 3 illustrates the weight percentage of the DNA whose N^{14} and N^{15} strands are separated as a function of hyperchromicity for 1, 2, and 4 per cent HCHO. The

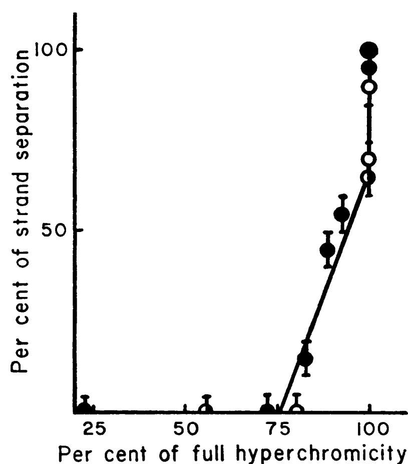

FIGURE 3 Percentage of the strand separation as a function of hyperchromicity in hybrid DNA heated for 10 minutes in solutions of 1 per cent (○), 2 per cent (◑), and 4 per cent (●) formaldehyde. The uppermost point (100 per cent strand separation) represents the superposition of results from many experiments in 1, 2, and 4 per cent formaldehyde.

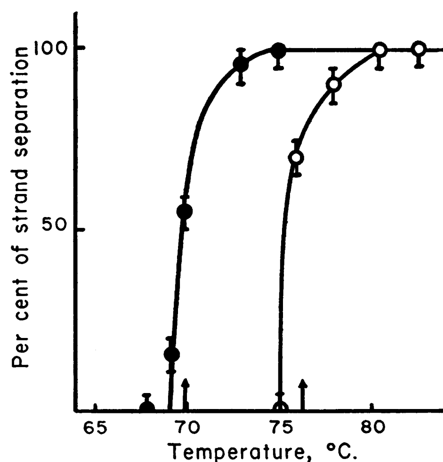

FIGURE 4 Relationship between the percentage of strand separation in hybrid DNA and the incubation temperature for samples heated in 1 per cent (○) or 4 per cent (●) formaldehyde for 10 minutes. The arrows indicate the onset of full hyperchromicity.

curve, within the limit of experimental error, is independent of HCHO concentration, *i.e.,* a given degree of separation corresponds to a specific hyperchromicity.

The same data is plotted in Fig. 4 illustrating the weight percentage of separated strands for two different HCHO concentrations as a function of temperature.

Melting-out curves of DNA in various concentrations of HCHO (obtained for constant time of heating) are invariant with respect to shape and are merely displaced to lower temperatures with increasing HCHO concentration (8). Therefore, since the two curves of Fig. 4 are essentially similar in shape but displaced along the temperature scale, one can deduce that separation is a function of hyperchromicity or degree of denaturation and not temperature or HCHO concentration alone. For instance, at 75°C there is complete separation in 4 per cent HCHO and no separation in 1 per cent HCHO.

Within the limit of detectability of the single strands (in these experiments about 5 per cent of the total material), there is no separation below about 75 per cent of full hyperchromicity, and 100 per cent hyperchromicity (within the limit of experimental error in optical density measurement) is not necessarily accompanied by 100 per cent separation. Although we have evidence that formaldehyde under certain conditions (8) can form cross-links between the strands, it is unlikely that formaldehyde is inducing these more heat-stable links for two reasons: (*a*) formaldehyde cross-links, when present, do not melt out even at 100°C, and (*b*) the percentage of the strands separating as a function of temperature is reproducible (Fig. 4), a result which would be most unlikely if the residual bonds were an experimental artifact. We may deduce that there exist in a substantial fraction of the molecules heat resistant cross-links, or regions, perhaps short, guanine-cytosine-rich, hydrogen-bonded sequences, which contribute negligibly to the hyperchromicity and which melt out within a few degrees above the shoulder of the melting-out curve. Geiduschek from other experimental results has independently arrived at the same conclusion (14).

We conclude from these experiments that the greater part of the width of a DNA melting-out curve represents intramolecular heterogeneity. The fact that 75 per cent of full hyperchromicity was reached before strand separation was detectable is of no profound significance. The presence of single-strand breaks along the molecule, occasioned by enzyme action during the DNA isolation procedure or by hydrolytic degradation during the heating for denaturation, would mean that single N^{14} or N^{15} strands would be detected before the complete unravelling of any one molecule. However, if the molecular weight of the preparation (6.5×10^6) were progressively lowered it is likely that the intramolecular heterogeneity would be translated to an intermolecular heterogeneity with probably little greater spread in the melting-out curve but a lowering of the temperature where complete strand separation commences.

It should be noted that in formaldehyde the extinction coefficient of denatured

DNA is approximately 143 per cent of that of native DNA whereas in the absence of formaldehyde it is nearer 130 per cent. Since the formaldehyde may not increase the absorbance contribution from the adenine-thymine and guanine-cytosine base pairs equally, the percentages of full hyperchromicity quoted in this paper are not necessarily equivalent to percentages measured in the absence of formaldehyde; moreover, the percentage of full hyperchromicity—in the presence or absence of formaldehyde—is not necessarily indicative of the degree of hydrogen bond breakage (15).

Meselson and Stahl (11) have shown that bacterial DNA is replicated semiconservatively and that the replicated subunits are separable from one another by heating to 100°C in 7.7 M CsCl. Cavalieri and Rosenberg (16) claimed that the DNA used in these experiments was 4-stranded and that the N^{14} and N^{15} subunits are double-stranded molecules. They further assumed that heating to 100°C in CsCl breaks the "biunial" bonds which hold the two double-stranded molecules together and that these bonds can be broken only by heating *in the CsCl.* However, we have found that heating the crude bacterial lysate to 100°C in the absence of CsCl also results in the appearance of the N^{14} and N^{15} bands. Further, Marmur and Ts'o (17) have separated the subunits by formamide denaturation at room temperature; Schildkraut, *et al.* (18) have reported the same separation in alkali and acid, and in 0.15 M NaCl − 0.015 M sodium citrate at temperatures below 100°C. These results and the present experiments clearly show that separation of the subunits demands neither CsCl nor 100°C. Our experiments do not indicate whether the subunits are single- or double-stranded molecules but do demand that the hypothesized biunial bonds break under conditions relating to the degree of hyperchromicity.

Our experiments show that separation of the subunits does not occur until approaching the top of the melting-out curve and is not complete until well past the temperature of the apparent onset of complete hyperchromicity. Clearly "full" hyperchromicity is an inadequate criterion of strand separation and hence complete denaturation. For example, heating to 100°C in ionic strength greater than 0.2 is not necessarily sufficient for complete separation of subunits in DNA with a high guanine-cytosine content, if these results obtained with *coli* DNA can be generalized.

This investigation was conducted in the laboratories of Professor Francis O. Schmitt for whose help and interest the authors are grateful. The authors also thank Mrs. K. Y. C. Ho for able technical assistance.

This investigation was supported by research grant E-1469 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States Public Health Service.

Dr. Freifelder was supported by a post-doctoral fellowship from the Division of General Medical Sciences, United States Public Health Service, 1961.

Received for publication, December 18, 1961.

REFERENCES

1. DOTY, P., MARMUR, J., EIGNER, J., and SCHILDKRAUT, C., 1960, *Proc. Nat. Acad. Sc.*, **46**, 461.
2. MARMUR, J., and DOTY, P., 1959, *Nature*, **183**, 1427.
3. SUEOKA, N., MARMUR, J., and DOTY, P., 1959, *Nature*, **183**, 1429.
4. ROLFE, R., and MESELSON, M., 1959, *Proc. Nat. Acad. Sc.*, **45**, 1039.
5. SHAPIRO, H. S., and CHARGAFF, E., 1960, *Biochim. et Biophysica Acta*, **39**, 68.
6. BURTON, K., and PETERSEN, G. B., 1960, *Biochem. J.*, **75**, 17.
7. DAVISON, P. F., FREIFELDER, D., HEDE, R., and LEVINTHAL, C., 1961, *Proc. Nat. Acad. Sc.*, **47**, 1123.
8. DAVISON, P. F., and FREIFELDER, D., 1962, in preparation.
9. BEER, M., and THOMAS, C. A., 1961, *J. Mol. Biol.*, **3**, 699.
10. GEIDUSCHEK, E. P., 1961, *Proc. Nat. Acad. Sc.*, **47**, 950.
11. MESELSON, M., and STAHL, F. W., 1958, *Proc. Nat. Acad. Sc.*, **44**, 671.
12. KIRBY, K. S., 1959, *Biochim, et Biophysica Acta*, **36**, 117.
13. BERNS, K. I., and THOMAS, C. A., 1961, *J. Mol. Biol.*, **3**, 289.
14. GEIDUSCHEK, E. P., 1961, private communication.
15. APPLEQUIST, J., 1961, *J. Am. Chem. Soc.*, **83**, 3158.
16. CAVALIERI, L. F., and ROSENBERG, B. H., 1961, *Biophysic. J.*, **1**, 317.
17. MARMUR, J., and TS'O, P. O. P., 1961, *Biochim. et Biophysica Acta*, **51**, 32.
18. SCHILDKRAUT, C. L., MARMUR, J., and DOTY, P., 1961, *J. Mol. Biol.*, **3**, 595.