PHYSICOCHEMICAL STUDIES ON THE REACTION BETWEEN FORMALDEHYDE AND DNA

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ABSTRACT The reaction between formaldehyde and phage T7 DNA has been studied by optical absorbance and sedimentation measurements. Through the course of denaturation, OD_{260} and $s_{20,w}$ rise; after the attainment of full hyperchromicity the $s_{20,w}$ falls sharply, suggesting a decrease in molecular weight. Conditions in which formaldehyde causes cross-linking are defined. Some experimental applications of the denaturation technique are given. Evidence which suggests that preformed single-strand interruptions may exist in phage DNA is briefly discussed.

INTRODUCTION

When DNA solutions are heated through a critical temperature range, the secondary structure of the molecules is disrupted, and, as a result of this denaturation process, the optical absorbance of the solution at 260 mμ increases. It has recently been shown that through the temperature range in which denaturation occurs, segments within molecules become disordered (1), and at any temperature an equilibrium state exists in which a certain fraction of each molecule is denatured (2). As the temperature is raised the intramolecular denaturation increases until a step ensues which, in DNA from higher organisms at least, is irreversible. This irreversible step has been interpreted as the separation of the two strands of the double helix (3).

Until the onset of this irreversible process, cooling a solution of partially denatured DNA molecules results in the recovery of the original optical absorbance and viscosity (4); hence the physical properties of partly denatured molecules can be studied only at the ambient temperature. However, at elevated temperatures hydrolytic degradation proceeds at a detectable rate (5). As an alternative to studies at high temperatures, the reassociation of the separated strands may be blocked by a suitable reagent such as formaldehyde. It has been shown that in the

presence of formaldehyde little or no decrease of optical absorbance ensues on cooling a partly or fully denatured DNA solution (6, 7), since the amino groups on the nucleotide bases react with the formaldehyde which thereby blocks the reformation of the hydrogen bonds dictating ordered reassociation of the strands (8).

In this paper we describe an investigation of the reaction between formaldehyde and a monodisperse DNA preparation from T7 bacteriophage (9). From these studies conditions which apparently eliminate strand hydrolysis have been defined. The conditions under which cross-linking occurs in the presence of formaldehyde have also been examined.

MATERIALS AND METHODS

1. *Growth of Phage and Preparation of DNA.* Bacteriophage T7 was grown as described elsewhere (9). Other phages were grown by similar techniques. Phage T4 was a shock-resistant mutant (10) which was purified by CsCl density gradient centrifugation. Phage DNA was prepared by a modification of the phenol technique (9).

Trout sperm and bacterial DNA were prepared by modified sodium dodecyl sulfate and phenol techniques (11, 12). Hybrid N^{14}—N^{15} *E. coli* DNA was prepared as previously described (1).

2. *Formaldehyde Solutions.* Reagent grade 37 per cent HCHO (Merck) was used for all experiments. This material contains 12 per cent methanol added as a polymerization inhibitor. The solutions were neutralized with about 0.1 ml of M NaOH per 10 ml HCHO, and 0.1 ml of molar phosphate buffer, pH 7.8, was added. Neutralization of HCHO causes an immediate increase in optical density at 260 mμ (OD_{260}) which continues for a period of several hours, the rate being temperature-dependent. To avoid errors resulting from a varying optical blank and to reduce the concentration of polymeric forms of formaldehyde, the neutralized 37 per cent stock HCHO solution was always boiled for 10 minutes in a sealed ampoule before use. No further OD_{260} increase occurs after boiling. Fresh solutions were prepared daily.

3. *Thermal Denaturation Curves.* In the absence of HCHO, DNA melting-out curves were obtained by heating the solutions in 3 ml stoppered cuvettes in the thermostated compartment of the Beckman model DU spectrophotometer.

Since in the presence of HCHO the hyperchromicity is both time- and temperature-dependent, it is clear that the melting-out curve must refer to a controlled time of heating. We chose to heat each sample for 10 minutes. The denaturing solution was chilled in iced water before addition of the DNA. For each point on the denaturation profile 1 ml of the DNA in the denaturing solution was sealed in a 2 ml glass ampoule and rechilled. The ampoules were then completely immersed in a constant temperature bath and after 10 minutes' heating quenched in iced water; the OD_{260} was read shortly afterwards.

4. *Denaturation Kinetics.* The time course of DNA denaturation at a given temperature was followed by adding a small volume of a concentrated DNA solution to a preheated denaturing solution in a covered cuvette in the thermostated compartment of the spectrophotometer.

For kinetic measurements requiring several hours, solutions were stored in sealed ampoules in constant temperature baths until the absorbance was measured.

For studies relating time of denaturation to the sedimentation properties, the denatur-

ing solution was placed in a screw-cap vial in a constant temperature water bath and heated for 15 minutes. The DNA solution—constituting less than 5 per cent of the volume of the solution—was then added. At various times samples were withdrawn with a pipette, quenched in small vials cooled in iced water, and subsequently studied in the ultracentrifuge.

5. *Alkaline Release of DNA from Phage.* (*a.*) *Denaturation by heating in formaldehyde.* To 0.67 ml of phage solution (ionic strength <0.002) was added 0.03 ml of M K_2PO_4. After 5 minutes at room temperature 0.05 ml M KH_2PO_4 was added, followed by 0.05 ml M phosphate buffer, pH 7.8, and 0.4 ml neutral 37 per cent HCHO. The solution was heated for 2 1/2 minutes at 70°C, cooled, and 0.4 ml 4 M NaCl was added before ultracentrifugation.

(*b*) *Denaturation by alkali.* To 0.05 ml of a phage solution (ionic strength <0.05) was added 0.05 ml M NaOH. After 1 minute at room temperature, 0.4 ml 37 per cent HCHO was added, followed by 0.1 ml M KH_2PO_4, 0.6 ml H_2O, and 0.4 ml M NaCl. The optical density of DNA treated in this way was unaffected by heating to 100°C for 10 minutes, a fact which indicates that the DNA had been fully denatured.

6. *Ultracentrifugation.* Sedimentation velocities were determined as described elsewhere (9). All solutions were diluted with one volume of 4 M NaCl to 3 volumes of the original solution. The final DNA concentration was always 20 ± 5 μg/ml. The sedimentation coefficients were corrected to 20°C and the viscosity of water ($s_{20,w}$), and are expressed in Svedbergs (S).

7. *Standard Denaturation Technique.* Of the several conditions that provided complete denaturation with minimal cross-linking and hydrolysis, the following standardized procedure was adopted for the final sedimentation studies. A solution containing 12 per cent HCHO and 0.1 M phosphate, pH 7.8, was preheated at 70°C for 5 minutes. The DNA (in a volume not greater than 1/20th of the denaturing solution) was added and, after heating for 2 minutes, the solution was quenched in iced water. If preheating was not feasible, heating was extended to 2 1/2 minutes.

RESULTS

1. *Optical Studies*

The relation between the OD_{260} of a DNA solution and temperature (the "melting-out" or denaturation curve) is a function of the composition of the DNA and of the pH, ionic strength, and composition of the solvent (13, 14).

Fig. 1 shows for three different HCHO concentrations the OD_{260} as a function of time after concentrated T7 DNA was added to preheated buffered HCHO solutions. The curves show that the rate of increase of OD_{260} rises with temperature and HCHO concentration.

The reaction at low temperatures is not shown in the graphs but does proceed at a measurable rate. For example in 4 per cent HCHO and 0.01 M phosphate at 25°C and 45°C, full hyperchromicity is reached in approximately 65 and 10 hours, respectively. These times are sufficiently long that at 25°C or lower, hyperchromicity can be considered as constant for the duration of most ultracentrifuge experiments.

Fig. 2 shows the DNA melting-out curves at four different concentrations of HCHO and at two salt concentrations. Two melting-out curves in the absence of HCHO, obtained by the usual procedures, are also given. In these cases a lower final hyperchromicity was attained (6). The maximum hyperchromicity is independent of ionic strength and HCHO concentrations within the range 1 to 12 per cent.

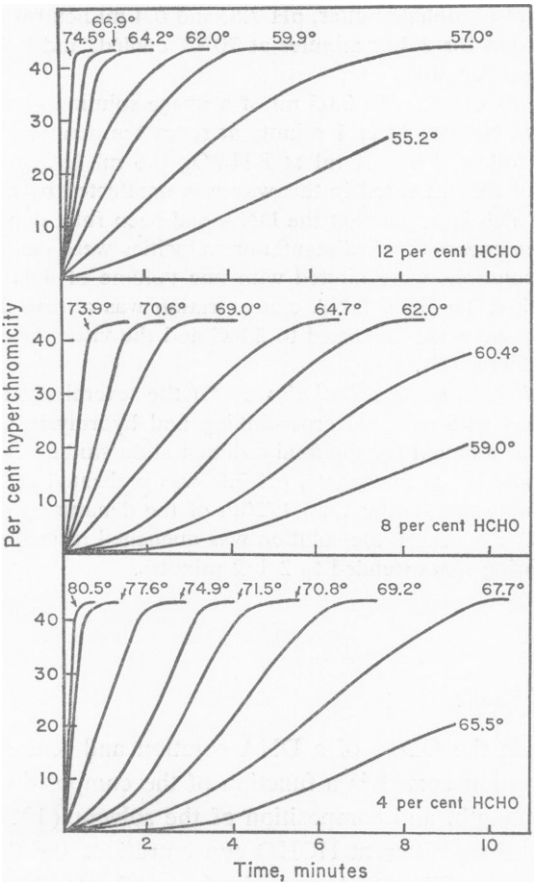

FIGURE 1 Percentage hyperchromicity as a function of time of incubation of T7 DNA in denaturing solutions at the temperatures indicated. The HCHO concentrations are given in the diagrams.

For constant heating time, denaturation occurs at lower temperatures with increasing HCHO concentration. Fig. 3*a* indicates for both salt concentrations employed the relationship between the HCHO concentration and the decrease in the temperature (T_m) at which the DNA is at 50 per cent of full hyperchromicity after 10 minutes' heating. The curves demonstrate that the decrease of T_m is not

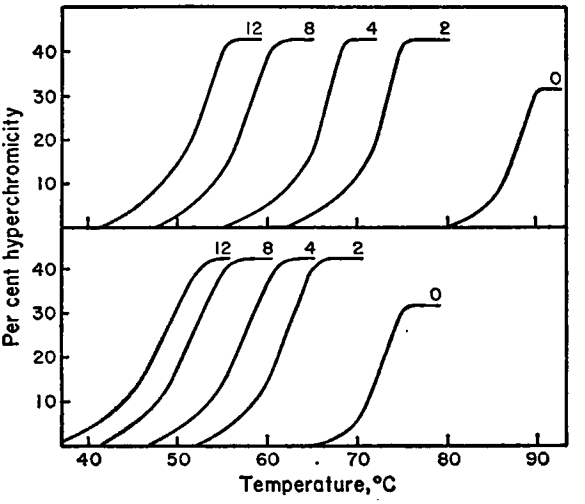

FIGURE 2 Melting curves for T7 DNA heated for 10 minutes in denaturing solutions containing HCHO in the percentages indicated. Upper curves, in 0.01 M phosphate, pH 7.8; lower, 0.1 M phosphate, pH 7.8.

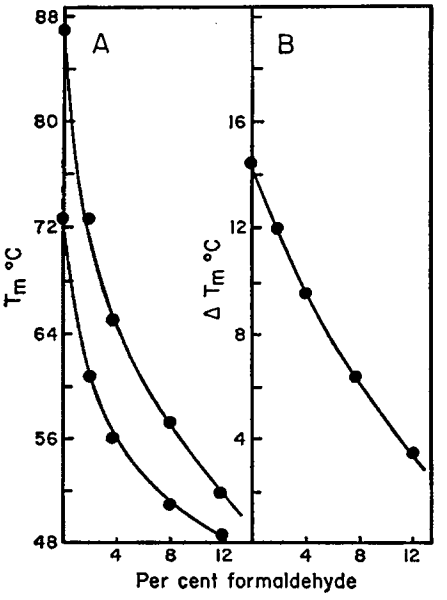

FIGURE 3A T_m obtained from the curves given in Fig. 2 as a function of HCHO concentration. Upper curve, 0.1 M phosphate, pH 7.8; lower, 0.01 M phosphate, pH 7.8.

FIGURE 3B Influence of salt on the denaturation temperature of T7 DNA in 0, 2, 4, 8 and 12 per cent, HCHO denaturing solutions. Each point represents the difference between T_m measured in 0.1 and 0.01 M phosphate, pH 7.8.

linear with respect to HCHO concentration. The curves for the two salt concentrations are not parallel, and a plot of ΔT_m (*i.e.*, $T_{m,0.1} - T_{m,0.01}$) *versus* HCHO concentration, as shown in Fig. 3*b*, indicates that with increasing HCHO concentration the effect of ionic strength on denaturation rate diminishes.

2. *Sedimentation Studies*

The transition from native to denatured DNA can be followed by sedimentation analysis of partially hyperchromic material. This is possible because the HCHO blocks the reformation of the hydrogen bonds disrupted during the heating period, yet the continued reaction of the native DNA with the HCHO is, after cooling, sufficiently slow to be ignored during the sedimentation experiment.

Native T7 DNA shows a sharp sedimenting boundary (see Fig. 1 in reference 9). The upper part of the trace shows a small curvature which is believed to be an artifact resulting from boundary disturbances and is not indicative of higher molecular weight material (9). This curvature appears on all the sedimentation diagrams (Figs. 4 and 8) and will be ignored in the following discussion. Partially or fully hyperchromatic T7 DNA shows a sharply defined leading component, followed by a fraction of more slowly sedimenting polydisperse material, the proportion of which increases with increased denaturation (Fig. 4). The sedimentation

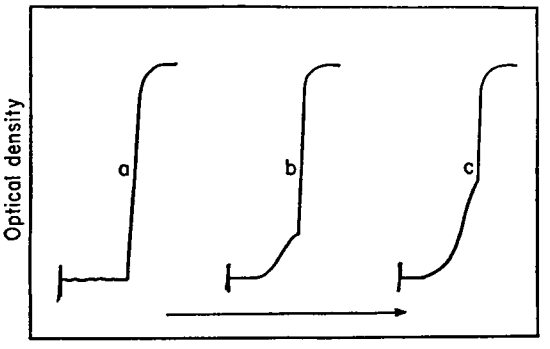

FIGURE 4 Photometric trace of UV absorption photograph of sedimenting T7 DNA denatured to different degrees.
a. 8 per cent hyperchromicity, median $s_{20,w} = 34.5$
b. 25 per cent hyperchromicity, median $s_{20,w} = 43.0$
c. 42 per cent hyperchromicity, median $s_{20,w} = 39.0$
The arrow represents the direction of sedimentation. The vertical bar at the left of each trace indicates the meniscus.

diagram is at all times biomodal, *i.e.*, the "tail" is not smoothly connected with the sharp boundary of the fast component. Since the slower material could have arisen from hydrolytic degradation under the denaturation conditions, attention was focused on the fast component, and the values of $s_{20,w}$ quoted in the following para-

graphs refer to that fraction of the DNA. Consideration of the slower sedimenting material will be deferred to the section dealing with hydrolysis.

Fig. 5 shows $s_{20,w}$ as a function of temperature for several T7 DNA preparations which had been heated in sealed vessels for 10 minutes in 12 per cent HCHO, 0.1 M PO_4, pH 7.8, and then quenched in iced water. Comparison with the melting-out curve shows that $s_{20,w}$ rises with increasing hyperchromicity, continues to rise when no further increase in OD_{260} is detectable, and then drops sharply.

In a few DNA samples heating under conditions which normally gave the fully hyperchromic 39S boundary gave instead a double boundary consisting of varying ratios of 39 and 65S material. With longer heating times or higher temperatures the 65S component decreased and the 39S increased. This anomalous behavior was ascribed to the presence of divalent ions in the DNA preparation since, when the denaturation was performed in the presence of 10^{-3} M versene, no double boundary was observed, *i.e.*, the DNA solutions behaved as if they contained one molecular species, each molecule undergoing the same change simultaneously at a unique temperature under these denaturing conditions.

The $s_{20,w}$ of the fully denatured material is greater than that of the native DNA, and with further heating it does not decrease until hydrolytic degradation is detectable.

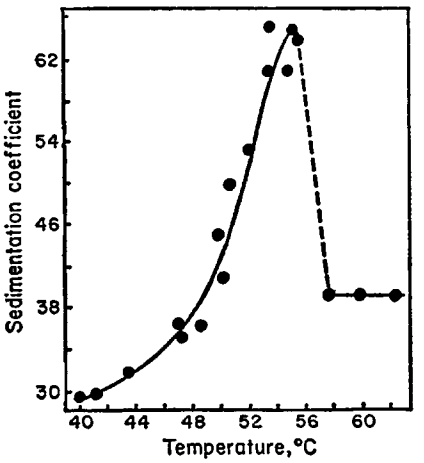

FIGURE 5 Sedimenation coefficient of the sharp boundary of T7 DNA fully or partly denatured by heating for 10 minutes at the indicated temperatures in 12 per cent HCHO, 0.1 M phosphate, pH 7.8.

The same data relating denaturation and $s_{20,w}$ is shown in Fig. 6 where the relationship between the percentage of full hyperchromicity and $s_{20,w}$ is plotted.

A similar relationship between $s_{20,w}$ and the progress of denaturation results when denaturation is carried out at constant temperature for various heating times. Fig. 7 shows the $s_{20,w}$ as a function of time after the addition of DNA to the denaturing solution preheated to 58.3°C. Again it is seen that $s_{20,w}$ increases with time and does not drop until past the onset of apparently full hyperchromicity.

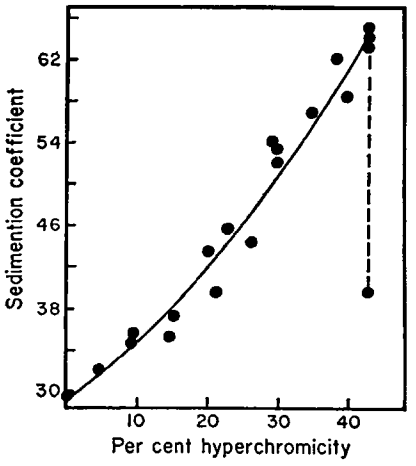

FIGURE 6 Sedimentation coefficient of the sharp boundary of T7 DNA, fully or partly denatured under several conditions, as a function of per cent hyperchromicity.

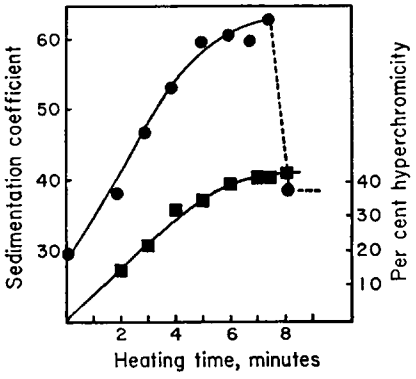

FIGURE 7 Sedimentation coefficient of the sharp boundary (•) and per cent hyperchromicity (■) of T7 DNA fully or partly denatured by heating at 58.3°C for the indicated times in 12 per cent HCHO, 0.1 M phosphate, pH 7.8.

3. *Hydrolysis*

As mentioned above, the percentage of T7 DNA in the slowly sedimenting "tail" of the sedimentation diagrams increases with hyperchromicity. In fully denatured DNA this tail includes material with $s_{20,w}$ ranging from 15 to 39 Svedbergs. That the percentage of DNA in the tail increased in old or degraded preparations suggested that some of this tail at least was an artifact; several experiments were therefore conducted to minimize the material in this tail. Careful control of the steps in the preparation and denaturation of the DNA brought the amount of material of lower $s_{20,w}$ down to 50 ± 5 per cent of the whole, and this percentage was observed consistently in 5 samples of DNA from 3 phage strains prepared by the phenol method.

To ensure that the 50 per cent tail was not a result of degradation brought about by the preparative or denaturation process, various modifications of these pro-

cedures were introduced. Two DNA preparations were made by alkali release; denaturation by the standard method again showed a 50 per cent tail. Two DNA samples, one prepared by phenol, the other by alkali release, were denatured in the following ways: (*a*) heating for 10 minutes at 60°C in 12 per cent HCHO, 0.1 M PO_4, pH 7.8; (*b*) heating for 2 minutes at 70°C in 12 per cent HCHO, 0.1 M PO_4, pH 7.8; (*c*) heating for 20 minutes at 40°C in 2 per cent HCHO, 0.2 M K_3PO_4, pH 11.6; (*d*) heating for 1 minute at 25°C in 0.5 M NaOH followed by addition of HCHO. For each of these samples the tail amounted to 50 per cent. After DNA was heated for 18 minutes at 70°C in 12 per cent HCHO, 0.1 M PO_4, pH 7.8, the tail on the sedimentation diagram was similar in shape and size to that of a sample heated for 2 minutes. However, when the DNA was heated for 30 minutes, the sedimentation diagram showed 80 per cent of the DNA in the slow tail; the tail also both lost its bimodality and gained very low $s_{20,w}$ material, presumptive evidence of hydrolysis.

These results suggest that the 50 per cent fraction of the DNA trailing the apparently homogeneous fast component is not an artifact. However, it may be deduced that any procedure which causes a loss of material from the fast component—with a concomitant increase in the tail—is producing breaks in the polymer strands. On the assumption that the fast component in the sedimentation diagram represents single polynucleotide strands half the molecular weight of the intact T7 DNA, the number of hydrolytic breaks in the single strands introduced by various conditions have been summarized in Table I. These numbers have been calculated on the basis of an empirical calibration of $s_{20,w}$ as a function of molecular weight for a series of formaldehyde-denatured DNA preparations; the derivation of this approximate relationship is presented below.

TABLE I

DEGREE OF DEGRADATON ACCOMPANYING VARIOUS CONDITIONS FOR DENATURATION

Treatment*	Median $s_{20,w}$	Per cent undegraded	Hydrolytic breaks/ 10^7 mol. wt. (±20%)
2 min. 70°C—12% HCHO	39	50	0
18 min. 70°C—12% HCHO	39	50	0
30 min. 70°C—12% HCHO	37	20	0.6
10 min. 75°C	34	10	0.8
10 min. 85°C	32	5	0.8
3 min. 100°C	34	10	0.8
3 min. 100°C	27	0	1.0
10 min. 100°C	26	0	1.0

* In each case in which HCHO was not present during heating, it was added to make 12% HCHO, and the solution was further heated for 2½ minutes at 70°C. For all experiments the solvent was 0.1 M phosphate, pH 7.8.

4. *Cross-Linking*

In certain circumstances the $s_{20,w}$ of the T7 DNA rises to the maximum value shown in Fig. 5, but with further heating or higher temperatures, $s_{20,w}$ does not decrease. If the drop in $s_{20,w}$ is interpreted as strand separation, then one can infer that the high $s_{20,w}$ material contains at least one heat-resistant interstrand cross-link. This cross-linking occurs when DNA is allowed to react with HCHO for long periods of time before heat denaturation. The degree of cross-linking can be determined by denaturing the DNA and measuring the amount of material sedimenting in the 65S peak. A sample with 25 per cent cross-linked material is shown in Fig. 8. Fig. 9

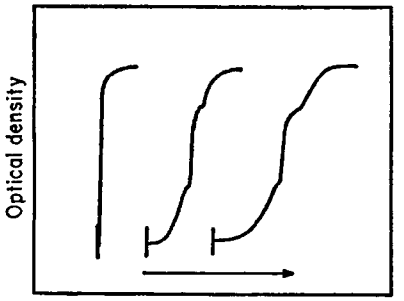

FIGURE 8 Photometric trace of UV absorption photographs of sedimenting T7 DNA taken at 0, 16, and 24 minutes afer reaching final speed of 33,450 RPM. The DNA was previously incubated in 12 percent HCHO, 0.1 M phosphate, pH 7.8, at 0°C for 11 1/2 hours, and then heated for 2 1/2 minutes at 70°C. Approximately 25 per cent of the strands are cross-linked. The arrow represents the direction of sedimentaion and the vertical bar at the left of each trace indicates the meniscus.

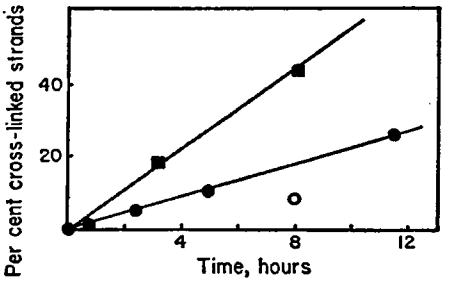

FIGURE 9 Percentage of cross-linked strands for T7 DNA pretreated (as described below) before heating in 12 per cent HCHO, 0.1 M phosphate, pH 7.8, for 2-1/2 minues.
- ● Incubated at 0°C in 12 per cent HCHO.
- ■ Incubated at 25°C in 12 per cent HCHO.
- ○ Incubated at 25°C in 3 per cent HCHO.

shows the percentage of cross-linked material as a function of time of incubation for various HCHO concentrations and temperatures. The cross-linking reaction is minimized at high pH (we are indebted to Dr. L. Grossman who pointed out this fact), *e.g.*, a sample treated for 24 hours at 25°C in 4 per cent HCHO, 0.04 M borate, pH 9, shows no cross-linking. Cross-linking is not confined to T7 DNA nor to intact molecules, since it has been observed in T2 DNA, sheared T2 DNA ($s_{20,w}$ = 25), *E. coli* DNA, and salmon sperm DNA. The cross-links are stable when briefly heated at 100°C. Cross-linking was also demonstrated and measured by storing N^{14}–N^{15} "hybrid" DNA from *E. coli* in HCHO and subsequently denaturing and banding in a cesium chloride density gradient.

5. *Further Denaturation Studies*

The standardized HCHO denaturation was performed on DNA released by alkali from T1, T5, and λ (*hc*) phages, and on T4 and T5 phage DNA obtained by phenol extraction. With T1 and λ (*hc*) a bimodal boundary consisting of a sharp region and a tail resulted, as is the case for T7. The $s_{20,w}$ for the sharp region was 35 for T1 and 39 for λ (*hc*). The percentage of material in the tail was 55 per cent for T1 and 28 per cent for λ (*hc*). The DNA of both T4 and T5 gave on denaturation a broad boundary with a median $s_{20,w}$ of 54 and 41, respectively; no sharp leading boundary was seen in the photometric traces. For each DNA the boundary was identical after heating for 2 or 4 minutes.

Curves similar to those in Figs. 5 and 6 relating $s_{20,w}$ to hyperchromicity or temperature have been obtained for DNA isolated from stationary phase *E. coli* and *Pseudomonas fluorescens*. The changes are not so sharply defined or so striking as for the T7 DNA (probably because of polydispersity), but still an increase in $s_{20,w}$ followed by a fall was observed through the denaturation range.

For both bacterial DNA's the viscosity of each sample used in the sedimentation studies was also measured. As the OD_{260} increased the viscosity decreased continuously showing no inflection over the range where the $s_{20,w}$ fell.

The sedimentation coefficient was also determined for sheared and sonicated T7 DNA and for intact and sheared trout sperm DNA. From these values a curve relating the $s_{20,w}$ of HCHO-denatured material and molecular weight has been derived in the following way: for trout sperm DNA the molecular weight was assumed to be half that of the native material, whose molecular weight was estimated from the empirical calibration of Doty, McGill, and Rice (15). Two further points were provided by the fast component of fully denatured intact T7 DNA (39S) and the maximum $s_{20,w}$ reached before strand separation (65S); the molecular weight of T7 DNA has been reported in another paper (9). The curve is given in Fig. 10.

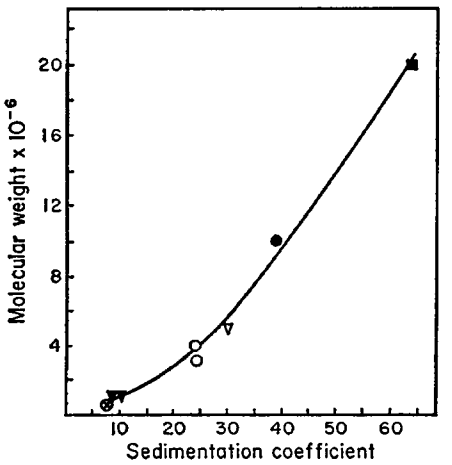

FIGURE 10 Relationship between $s_{20,w}$ and molecular weight for DNA denatured in HCHO.
⊗ Sonicated trout sperm DNA.
▼ Sonicated T7 DNA.
○ Sheared T7 DNA.
▽ Trout sperm DNA.
● Fully denatured T7 DNA.
■ Fully hyperchromic T7 DNA just prior to strand separation.

From the crudity of the derivation this curve is clearly suitable for only the most approximate calculations.

DISCUSSION

Some aspects of the denaturation of DNA in the presence of formaldehyde have been elucidated in the experiments reported above. The interpretation of the ultracentrifuge diagrams has been greatly facilitated by the use of a monodisperse DNA. With such a homogeneous material the physical properties of the solution reflect the molecular changes in each molecule, whereas the interpretation of experiments on more conventional DNA preparations is obscured by the physical and chemical heterogeneity of the molecules.

Under the conditions of these experiments the optical absorbance of a DNA solution in the presence of formaldehyde increased at a rate determined by the temperature, solvent, and formaldehyde concentration until the DNA was fully denatured. Grossman, *et al.* (6) have shown that the equilibrium reaction between formaldehyde and the amino groups of the bases heavily favors the hydroxymethylated form of the bases. Therefore, if it is assumed that in any DNA molecule native and "denatured" segments are, in the absence of formaldehyde, in thermodynamic equilibrium with hydrogen bonds constantly being made and broken—a likely contingency since the free-energy changes involved in the separation of the base pairs are in the order of kT (16)—then it is to be expected that in the presence of formaldehyde the amino groups will be progressively titrated until each molecule of DNA has been fully denatured.

At high formaldehyde concentrations the denaturation rate increases, as anticipated. This rate change may not arise solely from the changes in the equilibrium conditions, since Haselkorn and Doty have shown that formaldehyde, in destabilizing polyinosinic acid (17), must exert a denaturing effect distinct from its reaction with the amino groups. The methanol present in the formaldehyde solution may also increase the denaturation rate.

The change in $s_{20,w}$ of the fast component with hyperchromicity indicates that the optical changes occurring in denaturation are accompanied by physical alterations in the molecule. The hydrodynamic behavior of a macromolecule is a function primarily of its molecular weight and its shape, *i.e.*, frictional coefficient. The steady rise of $s_{20,w}$ is most simply interpreted as a progressive decrease in the friction coefficient of the molecules without any change in their mass. This would be the predicted behavior as the normally rigid DNA molecule assumed a more compact configuration as a result of the introduction of points of flexion at regions of hydrogen-bond rupture within the molecule, and the collapsing of the twin strands to a random coil configuration with the breakdown of hydrogen bonds at the ends of the molecules. Obviously with the progressive binding of the HCHO the weight

of the DNA also increases, but this factor probably contributes negligibly to the increase of $s_{20,w}$.

It seems unlikely that the formaldehyde can penetrate the DNA structure and react with the bases without the base pairs—and hence each polynucleotide strand —locally separating. This separation presupposes a degree of unwinding of the double helix. On the completion of denaturation and the hydroxymethylation of all the amino groups on the bases, there arises a question: Do the paired strands of the DNA separate or remain entangled? These studies show that after the DNA has become fully denatured, the sedimentation coefficient suddenly falls. This behavior could reflect a sudden extension of a compact molecule or a decrease in molecular weight. The former explanation appears unlikely, since a progressive fall in viscosity of *E. coli* and *Pseudomonas* DNA was observed through the denaturation range. We therefore interpret the change as a halving of the molecular weight of the DNA resulting from the physical separation of the strands.

It is of course possible that the subunits separating at the completion of denaturation are paired double helices (18), but since the separation appears to be related to the dissociation of base pairs (see also (1)) we presently prefer the postulate of strand separation.

It may be emphasized that this relationship between $s_{20,w}$ and denaturation can be observed only in the presence of a reagent blocking renaturation; in the absence of such a reagent a partly denatured molecule would return to the native configuration on cooling (2), whereas after strand separation the solution would contain a mixture of single strands, aggregated single strands, and renatured molecules.

It is clear that detectable cross-linking does not occur under the conditions finally chosen for denaturation. Although the chemical link has not been identified, it is possible that it is a methylene bridge between adenine groups, forming the adenine dimer described by Alderson (19). Intrastrand dimers, perhaps of adjacent adenines, may also be formed after denaturation. Such structures would not be detected in the present experiments but could interfere with renaturation of HCHO-denatured DNA from which the HCHO has been removed by dialysis.

It should be noted that "full" hyperchromicity is not an adequate criterion of complete denaturation or strand separation, as we have pointed out elsewhere (1). The strands cannot separate, we believe, until the last and strongest hydrogen bonds are broken; but these strongest bonds are a minute fraction of the total and contribute negligibly to the total hyperchromicity.

The significance of the low $s_{20,w}$ tail in the sedimentation diagram of denatured T7 phage DNA is obscure. A homogeneous DNA would be expected to yield a physically monodisperse denatured product; for this reason the sharply defined fast sedimenting component is believed to represent separated intact strands of the native DNA. It therefore seems evident that the slow "tail" comprises fragments of the DNA—moreover, the fragments are polydisperse, a fact which would appear

to rule out the possibility of a unique preformed break in one of the strands. Furthermore, from the bimodality of the sedimentation boundary, one can deduce that the loci for such breaks are not randomly distributed (20).

After full denaturation is achieved (2 minutes, 70°C), the sedimentation distribution is unchanged for heating times up to 20 minutes. Further heating results in both loss of material from the sharp boundary and the appearance of even slower material in the tail. Therefore, if this latter process represents phospho-ester bond hydrolysis, then the consistent appearance of a 50 per cent tail immediately upon denaturation—independent of the mode of DNA release or denaturation—demands the presence of preformed breaks or the rapid breakage of a very labile bond.

It is clear that the number of breaks is small, *i.e.*, since 50 per cent of the single strands are intact, either each phage DNA molecule contains one break or half of the molecules have no breaks and half have one in each strand. The absence of very slow material makes it unlikely that there are many molecules containing more than one or two breaks per strand.

Following the initial appearance of the 50 per cent tail, the kinetics of the loss of intact single strands with additional heatings shows a lag (20 minutes) and therefore represents a multi-event process. This process can be either the degradation of a multicomponent structure (18) by a single- or multi-hit mechanism or of a single component by a multi-hit mechanism, *e.g.*, if phospho-ester hydrolysis required preliminary depurination. These alternatives are not distinguishable in the present work. However, one can validly conclude that in the lag period there is hidden damage of some sort in the separated units.

If there exist preformed interruptions in the phage DNA strands, there should be some significance to the variation in the percentage of intact strands detected in the different phage species. Professor C. Levinthal has suggested that these interruptions might represent unrepaired chain breaks in molecules which have undergone pairwise exchange of double-strand material as proposed by Meselson and Weigle (21) for the mechanism of genetic recombination.

In earlier denaturation studies with formaldehyde, Berns and Thomas (7) concluded that there were no single-strand interruptions in T2 and T4 phage DNA. If their conclusion is true, then the broad sedimentation velocity distribution we observe for this DNA after denaturation must reflect a variety of molecular configurations rather than polydisperse fragments of the single strands. However, the mean $s_{20,w}$ of most DNA preparations we have studied increased on denaturation, suggesting that the separated strands have a $s_{20,w}$ greater than the parent DNA molecules. The fact that 25 per cent of the denatured T4 molecules have $s_{20,w} < 40$, *i.e.*, substantially lower than that of the undenatured molecules, strongly suggests that this material represents fragments of the single strands.

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