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[CONTRIBUTION FROM THE GIBBS LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

The Thermal Denaturation of Desoxyribose Nucleic Acid

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Several samples of thymus desoxyribose nucleic acid in saline solutions have been studied by light scattering, viscosity and sedimentation methods after exposure to a series of elevated temperatures. For exposure times of one hour, changes are noticed at temperatures above 89° for two of the samples and above somewhat lower temperatures for the others. From this characteristic temperature up to 100° the viscosity falls about 12-fold (as Zamenhof first noted), the mean end-to-end length decreases by about 15-fold and the molecular weight remains unchanged according to light scattering studies. Sedimentation measurements reveal a broadening of the distribution of sedimentation constants but no significant change in the average value. Typical denaturing agents lower the temperature range in which these changes occur by 10–20° but have no effect at room temperature. It appears that the changes at the elevated temperatures are due to the melting out of regions of hydrogen-bonded structure within DNA molecules and this coöperative breakdown of the highly organized, native structure is termed denaturation. At a given elevated temperature the viscosity quickly reaches an equilibrium value but it cannot be decided whether this indicates existence of a true equilibrium between native and denatured regions of the DNA molecules or a distribution of denaturation temperatures for different regions. It is shown that the completely denatured state can be achieved without chain degradation and it is surmised that in this state the polynucleotide strands are held together by remaining or reformed hydrogen bonds.

During the last three years it has become increasingly clear that desoxypentose nucleic acids (DNA) possess a uniquely ordered structure which is maintained in aqueous salt solutions but which can be irreversibly disordered by exposure to acid, alkali, heat and other agents. This breakdown of a specific structure is accompanied by a drastic fall in viscosity, a substantial rise in optical density in the ultraviolet and a shift to a different titration curve. Since the advent of the Watson–Crick structure for DNA it has been suggested frequently that this configurational change be identified with the breakdown of hydrogen bonding between the base pairs of the two polynucleotide chains.

When dissolved in 0.2 *M* NaCl, DNA can be exposed to *p*H values as low as 2.6 for several hours without significant chemical degradation. A study of the physical changes[1] showed that when brought to this *p*H the DNA molecules contracted without change in molecular weight. In this acid condition, the optical density at 258 mμ is raised about 32%.[2] A recent analysis of the titration curves of such acid–treated DNA shows that essentially all the original hydrogen bonds are broken if the temperature is 25° but that only about half are broken at low temperatures (0.4°).[3] It also has been shown that prolonged exposure to *p*H 2.6 at 25° leads to detectable chemical degradation: purines are liberated in substantial numbers, the polynucleotide chains undergo slow scission and the remaining hydrogen bonded areas disappear.[4]

The study reported here began in 1953 as a parallel investigation of the changes brought about in DNA by heat. Since the occurrence of a sharp fall in viscosity in a critical temperature range was readily apparent,[5] our first concern was to establish whether this was purely a configurational change as in the case of acid treatment or whether a molecular weight change was involved. In addition, we were concerned with characterizing the heat-treated DNA and elucidating the mechanism of the thermally induced transition. Further, these considerations led to an exploration of the problem of the separation of the two polynucleotide strands in DNA. Some results have been given in a preliminary note.[6]

(1) M. E. Reichmann, B. H. Bunce and P. Doty, *J. Polymer Sci.*, **10**, 109 (1953).

(2) R. Thomas, *Biochem. Biophys. Acta*, **14**, 231 (1954).

(3) R. A. Cox and A. R. Peacocke, *J. Polymer Sci.*, **23**, 765 (1957).

(4) C. A. Thomas and P. Doty, THIS JOURNAL, **78**, 1854 (1956).

(5) S. Zamenhof, H. E. Alexander and G. Leidy, *J. Exp. Med.*, **98**, 373 (1953).

(6) P. Doty and S. A. Rice, *Biochim Biophys. Acta*, **16**, 446 (1955).

Experimental

Samples.—Four preparations of calf thymus DNA were employed. Two of these have been described before in an earlier publication.[7] The sample listed there as Simmons B is designated here as SB-1 and the one listed as Varin is designated here as V-1. The third sample was prepared by Dr. Norman Simmons (University of California, Los Angeles) and is designated as SC-1. The fourth sample, on which most work was done, was prepared according to Simmons Method B and is designated SB-11. Briefly, this preparative procedure involves multiple extractions of minced thymus in saline–citrate (0.015 *M* sodium citrate and 0.15 *M* NaCl) by blending and centrifuging to obtain a sediment containing the nucleoprotein. 30% sodium *p*-xylene sulfonate is used to deproteinize the nucleoprotein. The protein is then precipitated upon dilution and filtered off. The DNA in solution is precipitated with isopropyl alcohol, redissolved and precipitated several times.

The molecular weight and radii of gyration as determined by light scattering are listed in Table I together with the intrinsic viscosity at zero gradient. The higher values for sample SB-11 are thought to be due to instantaneous transfer of the thymus gland from the freshly killed animal to Dry Ice and its remaining at this low temperature until blended in cold saline–citrate.

TABLE I

SUMMARY OF MOLECULAR PROPERTIES OF DNA SAMPLES

Sample	Mol. wt.	Radius of gyration, Å.	Intrinsic viscosity, 100 cc./g.
V-1	6,850,000	2050	48
SB-1	5,850,000	2000	53
SC-1	6,600,000	2700	62
SB-11	8,200,000	3000	72

Analytical Constants and Experimental Techniques.—The values of the extinction coefficient, refractive index increment, etc., are the same as those previously employed[7] and the techniques of light scattering and viscosity measurement are the same as described before.[7] In particular any viscosity measurement quoted implies that if gradient dependence were present, measurements in the range of 30 to 100 sec.$^{-1}$ were extrapolated to zero gradient to yield the value listed. Gradient dependence was found to disappear at intrinsic viscosities of about 10. Sedimentation measurements were made in a Spinco Model E ultracentrifuge using 30 mm. cells. Bar angles as low as 5° were used for the most dilute solutions. The saline–citrate solvent mentioned above (0.015 *M* sodium citrate and 0.15 *M* NaCl) was used throughout.

Experimental Results

Viscosity.—Preliminary measurements on all of the DNA samples confirmed Zamenhof's[5,8] observation that the viscosity of DNA solutions in physiological saline falls rapidly in the vicinity of 80°. A systematic study was carried out as follows. DNA solutions were made by dissolving the sample in water in the cold room to yield a concentration of about 40 mg./dl. This was adjusted to the standard saline–citrate solvent and a concentration of 4 to 7 mg./dl. Aliquots of this stock solution were heated at various temperatures by immersion in thermostats for one hour. The aliquots were then placed in ice and the viscosities measured within a day at 25°.

Results for samples V-1 and SB-11 are shown in Fig. 1. It is seen that the intrinsic viscosity of V-1 begins to fall at about 70° and reaches half its original value at 89°. Sample SB-1 showed a similar behavior. However, for sample SB-11 (and SC-1, not shown) the viscosity did not begin to fall until 85° and it reached half its value only at 94°. All samples reached about the same value of intrinsic viscosity at 100°, that is, 4.5 ± 1.5. The uncertainty is large here because of the high dilution employed in these particular measurements.

The relative sharpness of the viscosity fall in all four samples is indicative of a configurational change having the characteristics of a melting or denaturation of a periodic intramolecular structure. At first thought it appears that the process may involve only the passage of the individual DNA molecules from the original state of high viscosity to the final state of low viscosity. If it is assumed that the transition consists of a single first-order process, the data in Fig. 1 may be used to derive the temperature dependence of the first-order rate constant. When this is done, an activation energy of 35–40 kcal./mole is found for samples V-1 and SB-1 and a value of 93 kcal./mole for samples SB-11 and SC-1. Corresponding entropies of activation are calculated to be in the range of 63–75 entropy units for the first two samples and 220 e.u. for the SB-11 and SC-1 samples. These values are comparable with those found in protein denaturation studies and indicate that a highly ordered structure is being irreversibly broken down. Insofar as the term *denaturation* denotes the disappearance of a periodic structure by a rate process grossly characterized by a high entropy and high energy of activation, the transition described here can be termed denaturation.

The nature of the denaturation process was examined in more detail by observing the change in viscosity as a function of the time of exposure to elevated temperatures. Several results are shown in Fig. 2. The striking feature of these results is that they show that the viscosity falls rapidly and levels out at a plateau which is lower the higher the temperature. While it was not to be expected that the viscosity would measure precisely the extent of denaturation, except in the event of all-or-none reactions within individual DNA molecules, it clearly should give a qualitative indication of the progress of the reaction. Hence the leveling off of these curves demonstrates that the denaturation essentially comes to a stop short of completion by an amount dependent on the temperature. The implications which this observation carries are discussed later. However, at this point it is clearly evident that this observation invalidates the interpretation offered in the preceding paragraph.

The temperature range in which thermal denaturation occurs rapidly would be expected to be lowered by substances which are effective in denaturing proteins, as a consequence, measurements of the viscosity at 25° of a series of DNA solutions which had been heated at various temperatures for 1 hour were carried out in the presence of typical denaturing agents. The results for 8 *M* urea, 3.2 *M* guanidine hydrochloride and 0.8 *M* sodium salicylate are shown in Fig. 3 for sample SB-11. It is seen that the viscosity plots are approximately shifted to lower temperatures by these agents. The temperature at which the viscosity has fallen

(7) M. E. Reichmann, S. A. Rice, C. A. Thomas and P. Doty, THIS JOURNAL, **76**, 3047 (1954).

(8) S. Zamenhof, G. Griboff and N. Marullo, *Biochim. Biophys. Acta*, **13**, 459 (1954).

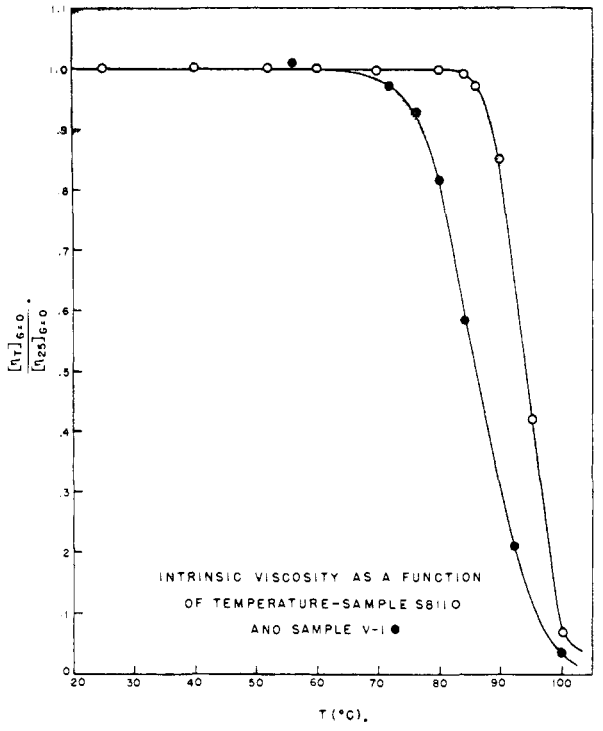

Fig 1.—Intrinsic viscosity of samples V-1 and SB-11 as a function of the temperature of one hour heating.

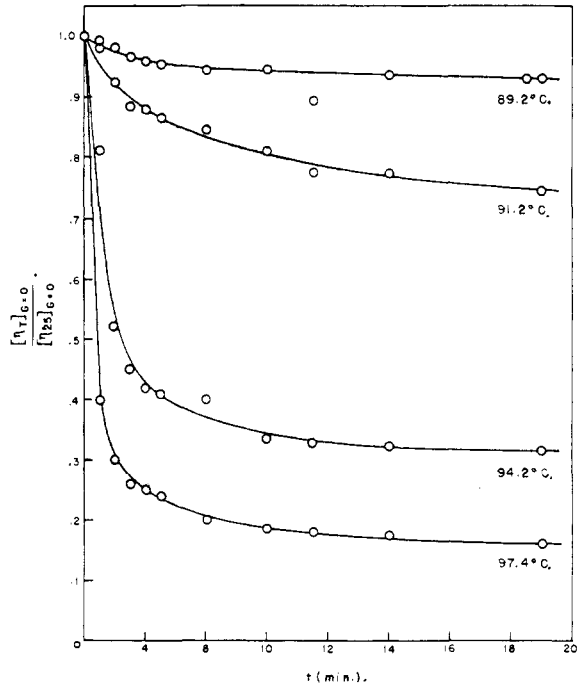

Fig. 2.—Intrinsic viscosity of sample SC-1 as a function of time at several temperatures.

by half is lowered 19, 14 and 8°, respectively, for the urea, guanidine hydrochloride and sodium salicylate. However, because of the difference in concentrations the molar efficiency of these agents is in the reverse order, similar to that usually found for proteins. It is also apparent that the viscosity of the denatured material is different in these various

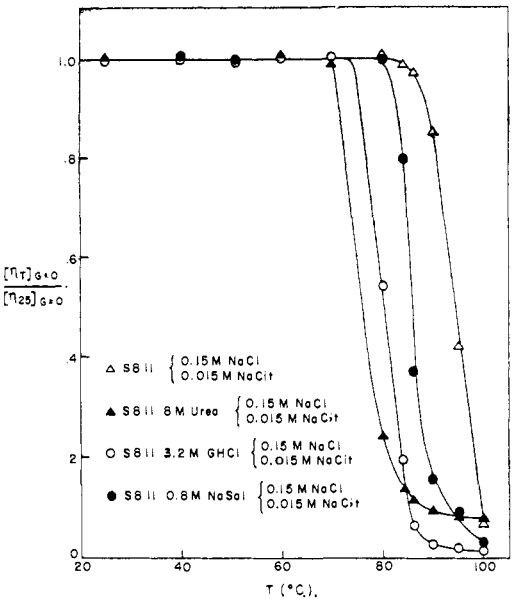

Fig. 3.—Intrinsic viscosity of sample SB-11 in the presence of hydrogen bond breaking agents as a function of the temperature of one hour heating.

solvents. It is lowest in the guanidine hydrochloride; this is probably due at least in part to the suppression of the electrostatic repulsions among the phosphate groups by the guanidinium ion. Separate experiments have also established that the molecular weight of DNA after exposure to 3.2 *M* guanidine hydrochloride at 100° for one hour is lowered to 940,000, which, as we shall subsequently see, is markedly lower than after comparable treatment in the absence of any denaturing agent. It is important to note that the DNA solutions employed here always contained 0.15 *M* NaCl and 0.015 *M* sodium citrate before the denaturing agent was added. The addition of any of these denaturing agents did not significantly change the intrinsic viscosity, 72. Hence the only observed effect of these agents, provided that they are added to saline solutions of DNA, is to lower by as much as 20° the denaturation temperature range of DNA. They have no effect at room temperature.

These conclusions stand in apparent conflict with the investigations of Conway[9] for he has found that 6 *M* urea raises the intrinsic viscosity (at zero shear) of aqueous solutions of DNA and lowers it substantially in the presence of 0.1 *M* NaCl. Since these observations appear reliable and the intrinsic viscosity of the DNA sample, 45, is high enough to ensure that it is not seriously denatured, it appears that the fall in viscosity in the presence of 0.1 *M* NaCl could only have come about if the urea was added before the salt. From the work of Thomas[2] and that of M. Litt[10] in this Laboratory it is clear that the denaturation temperature range is lowered markedly as the ionic strength is reduced so that in the absence of added salt and at rather high dilution denaturation takes place in the vicinity of room temperature. The effect of adding urea and other denaturing agents under this condition will

(9) B. E. Conway, *J. Polymer Sci.*, **20**, 299 (1956).
(10) M. Litt, private communication.

clearly be to accelerate the denaturation that is on the verge of occurring in any event. Thus the conflict between Conway's observations and our own is removed if our conclusion is stated in this form; concentrated urea lowers the denaturation temperature range of DNA by approximately 20°. In the absence of salt this brings it below room temperature while in the presence of salt (0.1 *M* or more) it remains well above room temperature. This view is also consistent with the observation that urea has no effect on the transforming activity of Pneumococcus DNA[11] since 0.1–0.2 *M* salt is generally present to protect it from thermal denaturation.

Light Scattering.—The interpretation of the thermally induced viscosity changes depends on what changes, if any, occur in the molecular weight as a result of the exposure to elevated temperatures. Light scattering measurements provide average values for both molecular weight and radius of gyration (a measure of size) when properly carried out[7] and consequently an adaptation of a light scattering cell was made that would permit angular intensity measurements to be made on the same DNA solution before and after heating without removal from the cell. This was done by sealing a sintered glass frit (ultra-fine) to a tapered glass joint (female) which could then be fitted onto the joint at the top of the light scattering cell which was itself shaped like an erlenmeyer flask. The glass frit prevented dust from entering the cell during the adjustment of pressure that took place on heating and cooling.

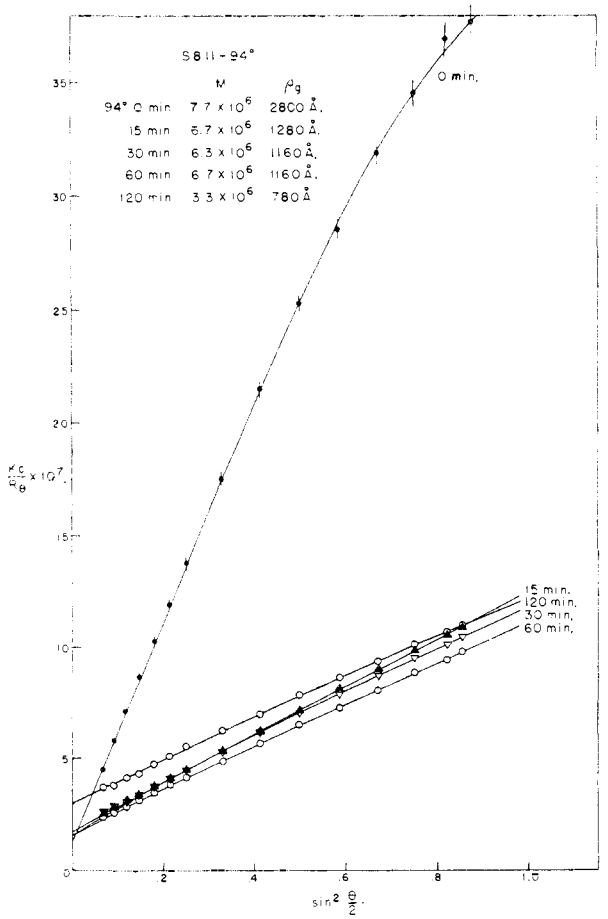

Fig. 4.—Angular distribution of scattered light for sample SB-11 heated at 94°.

Solutions of about 6 mg./dl. DNA (sample SB-11) in the saline–citrate solvent were then measured at 25° as a function of time exposed to temperatures of 84, 94 and 100°, respectively. In this concentration range the scattering is proportional to the concentration so that the molecular weight can be obtained from measurements at a single concentration. The temperature of 84° induced no change in scattering (and hence no change in molecular weight or size) until after 10 hr. (At 21 hr. the molecular weight had fallen to one-half and the radius of gyration had diminished about 25%.) This display of thermal stability was not unexpected since the viscosity had not fallen after one hour at this temperature. At 94 and 100°, however, substantial changes occurred as shown in Figs. 4 and 5 where the reciprocal scattering envelopes (Kc/R_θ) are plotted as a function of $\sin^2(\theta/2)$ (θ is the scattering angle and R_θ the reduced intensity of light scattered at θ). In both cases the slope of the plot falls abruptly while maintaining essentially the same intercept showing thereby that the molecular size is greatly diminished while the molecular weight remains approximately con-

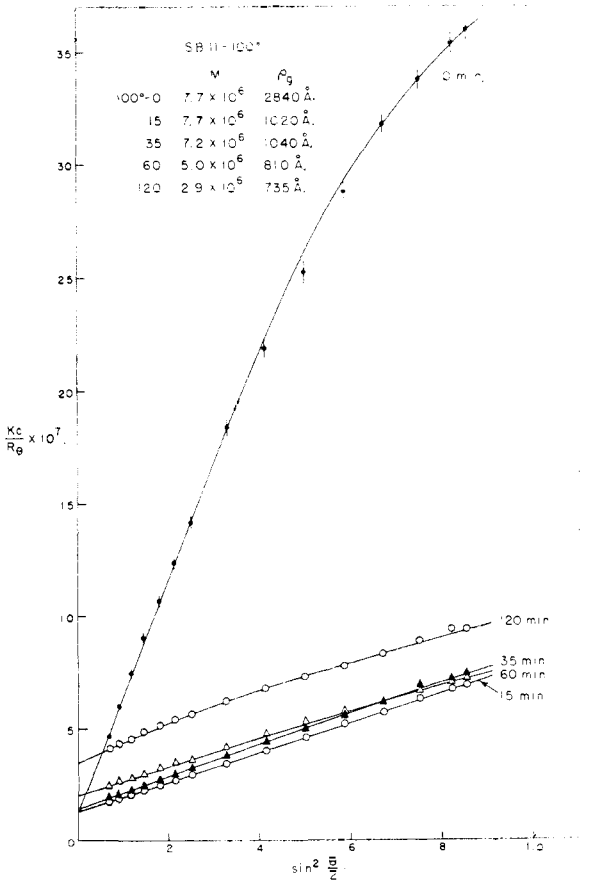

Fig. 5.—Angular distribution of scattered light for sample SB-11 heated at 100°.

(11) H. Ephrussi-Taylor, "McCollum–Pratt Symposium on the Chemical Basis of Heredity," Johns Hopkins Press, 1957, p. 299.

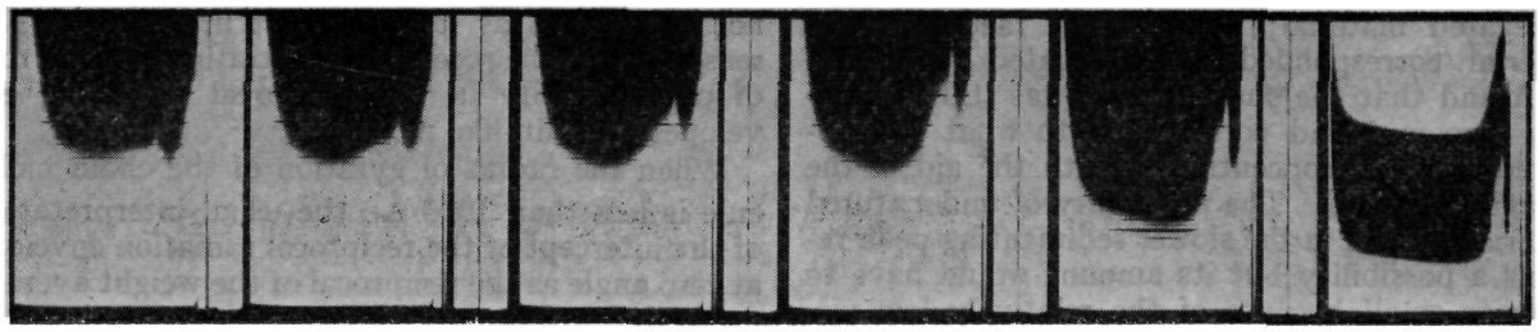

Fig. 6.—Schlieren photographs of sample SB-11 heated at 100° for 20 min. The photographs are at 4 minute intervals, the rotor speed is 37020 r.p.m. and the bar angle is 15, 7.5, 5, 5, 5 and 5° reading from right to left. The DNA concentration is 0.0429%.

stant. At longer times the molecular weight does decrease as shown by the upward trend of the intercepts.

These results show that thermal denaturation, like the case of acid denaturation summarized in the Introduction, consists of a substantial contraction of the molecule without a change in molecular (or particle) weight. In keeping with the viscosity results shown in Fig. 2 the radius of gyration falls from 2800 Å. to an intermediate and essentially constant value of 1250 Å. at 94° whereas at 100° it falls quickly to a lower value of 1000 Å. At both temperatures two hour exposures cause degradation to an average molecular weight of about 3 million and a radius of gyration of about 750 Å. Thus thermal denaturation appears to reach its final extent at a given temperature rather quickly and is then followed by a slow degradation.

In this case as in that of acid denaturation, there is no evidence of a lowering of the molecular weight to one-half as might be expected if the two polynucleotide strands separated. This indicates that either the breakdown of the hydrogen bonding is incomplete or that other types of bonds form which prevent the separation. Further consideration of this problem is taken up in a later section.

The attainment of a stable, intermediate value of both viscosity and size from light scattering when the solutions are exposed to temperatures lower than that required to produce the maximum effect raises a fundamental question. Have all the DNA molecules partially denatured or have some become completely denatured and others remained unaffected? In other words, do different parts of the individual DNA molecules have different resistances to thermal denaturation or is there a spectrum of melting temperatures among the population of DNA molecules?

In an attempt to answer this question and to characterize further the denatured state, some sedimentation studies were undertaken.

Sedimentation.—The sedimentation experiments were carried out in a manner similar to the light scattering experiments except that concentrations of 35 mg./dl. rather than 10 mg./dl. were employed. After heating at various times up to six hours at 84° no change in the sedimentation pattern or rate was observed over that of the unheated material. Heating at 94° for one hour brought about a slight broadening in the sedimentation pattern but it still remained hypersharp. The observed sedimentation constants (11.8 S. at 35 mg./dl. and 8.5 at 70 mg./dl.) are not much different than for the unheated material. In view of the previous evidence that a state of incomplete denaturation is reached under these conditions it appeared possible that the more denatured portion of the sample could not be resolved from the relatively undenatured material under these conditions. To test this possibility, synthetic mixtures of native DNA and DNA denatured at 100° were mixed and it was found that separation did not occur until the relative concentration of the native DNA had been reduced to nearly 10%. Hence the observed sedimentation diagrams of the 94° material could not discriminate between the two possibilities mentioned at the end of the preceding section.

When DNA solutions exposed to 100° for 15 or 20 minutes were examined it was found that half of the material appeared as a rather sharp, but not hypersharp, peak and that the remainder sedimented faster as a very diffuse shoulder that eventually separated (see Fig. 6). The pattern did not differ significantly over the concentration range of 35 to 53 mg./dl. In each case the sedimentation rate of the sharper peak was about 21 ± 2 and the diffuse peak extended from about 25 to 40 S.

The appearance of two differently sedimenting species in solutions in which it was thought that denaturation had gone essentially to completion posed a difficult problem. We had originally interpreted it as incomplete denaturation and associated the diffuse peak with the completely denatured material. This was the basis of the assignment of 30 as the sedimentation constant of the denatured DNA in the preliminary note.[6] However, quite recently, Dr. K. V. Shooter and Professor J. A. V. Butler have examined our sample SB-11 by ultraviolet optics in the ultracentrifuge[12,13] and found that the distribution of sedimentation coefficients broadened but remained single-peaked at 21 S. when heated at 100° for 15 min. at a concentration of 10 mg./dl.

Now it is well known that when DNA is thermally denatured at higher concentrations (>100 mg./dl.) the initial fall in viscosity is gradually restored and even surpassed. This presumably is due to the formation of intermolecular hydrogen bonds making use of the large number of sites opened up in the denaturation process. When this is taken together with the absence of the double peak at 10 mg./dl. it appears possible that our observation at intermediate concentrations was made on partially

(12) K. V. Shooter and J. A. V. Butler, *Trans. Faraday Soc.*, **52**, 734 (1956).

(13) K. V. Shooter, R. H. Pain and J. A. V. Butler, *Biochim. Biophys. Acta*, in press.

aggregated material; that is, the faster, diffuse material corresponded to aggregated denatured DNA and that the sharper peak was still molecularly dispersed and corresponds to what was observed at lower concentration with the aid of the ultraviolet optics. The possibility of undenatured material staying in the slower sedimenting peak remains a possibility but its amount would have to be quite small because of the relatively low viscosity.

The Denatured State

Molecular Weight.—From the results presented in Figs. 4 and 5 it appears that the exposure of DNA in saline solution to temperatures of 94 and 100° produces denaturation (as seen in the lowering of the slopes) followed by the gradual onset of chemical degradation (as seen in the eventual rise in intercepts). At 94° the slope, and hence the radius of gyration, is not lowered as much as at 100° in keeping with the viscosity results (Fig. 2) which shows that denaturation proceeds only to an intermediate point at 94°. The fact that there is a delay in the onset of the fall in molecular weight after completion of the denaturation is seen in Fig. 7 where the light scattering molecular weight results for samples SB-11 and V-1 are plotted as a function of time. The denaturation, judged either in terms of viscosity or light scattering, is essentially complete at 15 min. but the initial fall in molecular weight is not evident until about 45 min. The fall in molecular weight continues as long as the sample is held at the elevated temperature, and it exhibits no leveling off at any particular value.

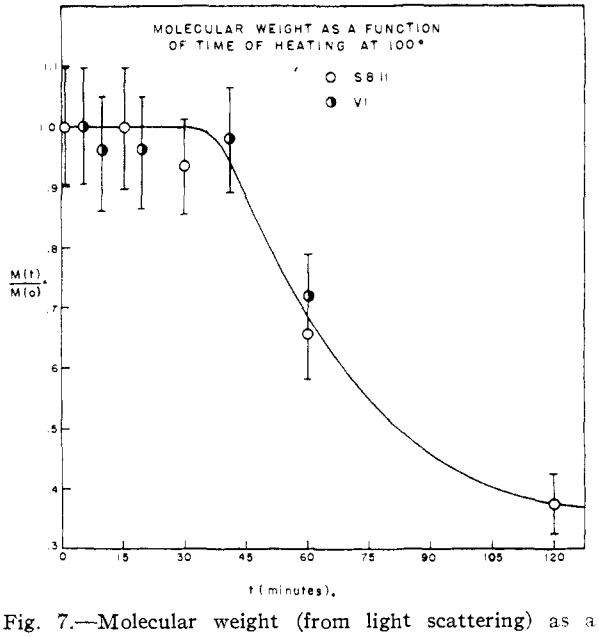

Fig. 7.—Molecular weight (from light scattering) as a function of time of heating at 100°.

In the case of thermal denaturation studied here as well as in the case of acid denaturation[1] the denatured material, prior to its eventual degradation, exhibits the same intercept in the light scattering plots as the original sample. Although we do interpret this as showing that denaturation occurs without change in molecular weight the conclusion is not as obvious as it appears at first sight. The reason lies in the recent demonstration[14] of the role of polydispersity in the reciprocal scattering envelopes of chain-like molecules.

When the radius of gyration of the chain molecule is less than 1000 Å., the usual interpretation of the intercept of the reciprocal radiation envelope at zero angle as the reciprocal of the weight average molecular weight is correct. However, when the radius of gyration exceeds 1000 Å., the reciprocal scattering envelope that is experimentally accessible will not yield the weight average molecular weight unless the molecular weight distribution happens to be the most probable (*i.e.*, one in which the various molecular weight averages stand in the ratios $M_z:M_w:M_n = 3:2:1$). When the molecular weight distribution is not of this form, the interpretation of the experimental data must be modified. For these other cases, the reciprocal radiation envelope will either approach or coincide with an asymptote whose intercept on the ordinate provides the reciprocal of two times the number average molecular weight, M_n. The implications of this possibility of ambiguity in assigning the type of average that is being determined by light scattering measurements on DNA is discussed elsewhere.[15] It is sufficient to say here that several lines of evidence indicate that the distribution of molecular weights in DNA does correspond roughly to that of the most probable distribution. If we call the molecular weight obtained from the scattering measurements in the usual angular range the "light scattering" molecular weight, we may conclude that in the present case the weight average molecular weight of denatured DNA is within probable experimental error equal to the light scattering average molecular weight for undenatured DNA.

Sedimentation Constant and Intrinsic Viscosity of Denatured DNA.—The intrinsic viscosity of thermally denatured DNA has been found in this study to be 4.5 ± 1.5. This is in need of more accurate and precise determination, but it is sufficiently reliable for the present purpose which is to explore the interpretation of this quantity in combination with the sedimentation constant. The work of Professors Butler[13] and Schachman[16] and their colleagues indicates that there is a rather broad distribution of sedimentation constants in DNA and that this broadens further upon thermal denaturation. On sample SB-11 the value determined at 10 mg./dl. (20.0 S) upon correction for concentration dependence becomes 21 S. Taken at face value, these measurements are in apparent contradiction with the viscosity and light scattering results found in this research. The constant value of the sedimentation constant would imply that two molecules with the same mass but which differ by a factor of three in radius of gyration (a factor of 27 in molecular volume) sediment at the same

(14) H. Benoit, A. M. Holtzer and P. Doty, *J. Phys. Chem.*, **58**, 635 (1954).

(15) P. Doty, The Physical Chemistry of DNA, Oak Ridge Symposium on Physical Chemistry of Biocolloids, April, 1956 (to be published).

(16) V. N. Schumaker and H. Schachman, *Biochim. Biophys. Acta*, **23**, 628 (1957).

rate. Since the chemical composition remains unchanged, the interactions of the individual monomers with the solvent should be of the same order of magnitude in both cases, and if we allow the denatured molecule to have a frictional coefficient more in accord with that characteristic of the single polynucleotide chain, the net resistance to flow per monomer must decrease. Even if the frictional coefficient per monomer were to remain the same, the large decrease in molecular volume at constant molecular weight would of necessity be accompanied by an increase in sedimentation rate. We can invent no mechanism which would enable a constant sedimentation constant to be coupled with a constant molecular weight and a greatly diminished molecular volume. The use of the sedimentation data of Professors Butler and Schachman with our molecular weights and viscosities also stand in contradiction with the empirically well established Flory–Mandelkern relation[17]

$$s_0 = 2.6 \times 10^6 \frac{M^{2/3}}{[\eta]^{1/3}} \frac{(1 - \bar{v}\rho)}{\eta_0 N}$$

In order to be compatible with the observed molecular weight of 8.2×10^6, the observed value of the sedimentation constant would have to be increased by a factor of 2.3.[18]

The problem posed by this large discrepancy is a serious one. The result obtained from sedimentation and viscosity is subject to several criticisms. First, so little is known about the molecular form of the denatured state that the application of the foregoing equations based upon simple models may not be justified. Secondly, the distribution of sedimentation constants, particularly in the denatured material, appears to be so broad that the weight average sedimentation constant, which is the one required in the above relations, may not have been properly determined. Thirdly, as we have pointed out previously,[6] the free draining coil model does permit a contraction of a chain-like molecule with only a small change in sedimentation constant but large decrease in viscosity. The relationship between s, $\overline{r^2}$, and $[\eta]$ is, in this model

$$s_0 = \frac{(1 - \bar{v}\rho)\overline{r^2}}{3600\eta_0[\eta]}$$

which predicts a value for s_0 of 20, in substantial agreement with experiment. However, this model does not fit the sedimentation data for native DNA as well, and the native molecule would be expected to be even more "free draining." Moreover, its applicability to non-homologous molecules may not be justified. Admittedly, the applicability of this model has been very limited but it may possibly be relevant in this case. On the other hand, the light scattering results are not dependent upon a model for their interpretation. With regard to the light scattering experiments it appears that aside from gross experimental errors the only complication that could account for the difference indicated here would be the existence of some aggregates having the same spacial distribution and radius of gyration as the individual denatured DNA molecules. These aggregates would have to have such large sedimentation constants, however, that they would for the most part be removed by the usual centrifuging employed in clarifying light scattering solutions. Such effects were not observed.

In our opinion, then, the light scattering results should be accepted but this dilemma cannot be considered satisfactorily settled until the sedimentation and viscosity data are rigorously interpreted.

Other Characteristics of the Denatured State.—In Fig. 8 the titration curves of undenatured and denatured (15 min. at 100°) DNA with dilute HCl (0.00494 N) are shown. The change produced upon thermal denaturation is similar to that found for exposure to acid[19] and in thermal denaturation studies being currently reported by Cox and Peacocke.[3] This agreement supports the view that essentially the entire hydrogen-bonded structure is destroyed by the thermal denaturation at 100°.

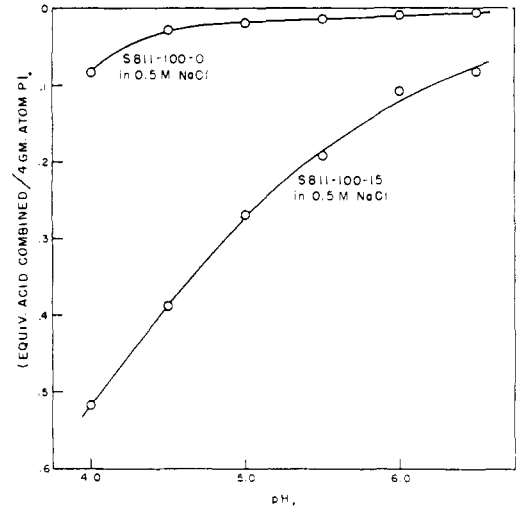

Fig. 8.—Potentiometric titration curve of samples SB-11 in 0.5 M NaCl before and after heating at 100° for 15 min.

If the hydrogen-bonded structure is essentially eliminated by the denaturation, it would be expected that the resulting molecule would be quite flexible in contrast to undenatured DNA. It is well known that typical polyelectrolyte molecules expand when the ionic strength is reduced and that this results in an increase in the reduced specific viscosity. That denatured DNA behaves in this manner while undenatured DNA does not is shown in Fig. 9 where the reduced specific viscosity is shown for solutions of each. In this experiment a portion of a DNA solution in saline–citrate (ionic strength = 0.165) was thermally denatured and then each solution was diluted stepwise with water producing thereby a continuous diminution in ionic strength. The rise in reduced specific viscosity in the case of the denatured DNA shows that its flexibility is comparable to that of ordinary flexible polyelectrolytes. The constancy shown

(17) L. Mandelkern and P. J. Flory, *J. Chem. Phys.*, **20**, 212 (1952).

(18) The use of a value of 2.6×10^6 in the foregoing equation has been shown to imply a very narrow molecular weight distribution [M. L. Hunt, S. Newman, H. A. Scheraga and P. J. Flory, *J. Phys. Chem.*, **60**, 1278 (1956)]. However, if the distribution were as broad as the most probable distribution ($M_z:M_w:M_n::3:2:1$) the calculated value of s_0 would still be 2.1 times that observed.

(19) R. A. Cox and A. R. Peacocke, *J. Chem. Soc.*, 2499 (1956).

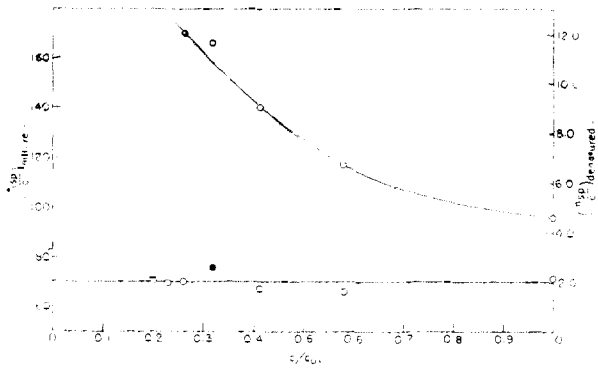

Fig. 9.—The expansion of denatured (15 minutes at 100°) DNA as a function of decreasing ionic strength as shown by η_{sp}/c.

by undenatured DNA reflects its rigidity. The experiment was conducted in the region of salt concentrations where intermolecular interactions are suppressed. At still lower ionic strengths these intermolecular interactions dominate and would mask any polyelectrolyte expansion.

Our conclusions on the state of thermally denatured DNA may be summarized as follows. DNA molecules in saline solution undergo a pronounced contraction when the temperature is raised above a critical value. This is accompanied by a large decrease in intrinsic viscosity but no attending change in molecular weight according to light scattering criteria. However, continued exposure to the elevated temperature does result in the onset of a degradative reaction with accompanying molecular weight decay. It appears that there is a distribution of critical denaturation temperatures among the DNA molecules because at a given temperature within a range (88 to 100° at 0.165 ionic strength) the viscosity falls quickly to a limiting intermediate value. This is interpreted as meaning that some molecules denature completely while others remain essentially unaffected. At 100° (and 0.165 ionic strength) the denaturation of all the DNA molecules appears to be complete in 15 min. and no change in molecular weight is evident. This condition is referred to as the completely denatured state. The extinction coefficient at the 259 mμ peak is 15% greater than before denaturation. The intrinsic viscosity is reduced by a factor of about 15 and the radius of gyration by a factor of 3. The sedimentation constant is apparently not greatly changed although this point in particular deserves further study. When the denaturation is carried out at higher concentrations than those employed here, reaggregation is apparent. This indicates a potential associating tendency of the denatured molecules that may only be operative intramolecularly at the higher dilution used in this work.

The Denaturation Process.—The course of the transition from the native to the denatured state has been followed principally by viscosity studies as illustrated in Figs. 1 and 2. If it is assumed that the intrinsic viscosity is a direct measure of the extent of the denaturation reaction and that the measurements made upon cooling to room temperature do represent the state of affairs existing at the temperature prior to cooling, two deductions are possible.

One of these recognizes that the viscosity has fallen to an equilibrium value within the time of one hour heating and that an apparent equilibrium constant can then be evaluated by interpreting the fractional lowering of the intrinsic viscosity in terms of fractional conversion of the native to the denatured state. (This interpretation of the data of Fig. 1 in terms of an equilibrium constant replaces the kinetic treatment mentioned previously.) When this is done for sample SB-11 a plot of the logarithm of the apparent equilibrium constant against the reciprocal of absolute temperature is found to be linear with the exception of the 100° point where it is known that substantial degradation has occurred. From the slope of this plot a heat of reaction of 110 kcal. and an entropy of reaction of 300 e.u. is derived. The data obtained in the presence of denaturing agents (Fig. 3) is fitted with the same heat of reaction and slightly higher entropy values (308 to 319 e.u.). Similar results are found for sample SC-1; for samples SB-1 and V-1 the values obtained are only one-third of those for the other two samples. These values emphasize that the denaturation reaction involves very large heat and entropy changes without chemical bond scission and in addition these two quantities offer a means of formally characterizing the course of the denaturation when it is carried through a series of temperatures with equilibration permitted at each temperature.

The other possible deduction relates to the kinetics rather than the equilibrium aspect of the reaction. If again the viscosity is assumed to represent quantitatively the progress of the reaction, the initial slope of the viscosity–time plots such as those in Fig. 2 can be subjected to the orthodox treatment of rate constants. When this is done for sample SC-1, the results are found to be linear in an Arrhenius plot (log of rate constant against reciprocal of absolute temperature) and from this an energy of activation of 145 kcal. and an entropy of activation of 325 e.u. is found.

Even if the assumptions on which these numerical results have been obtained are accepted their interpretation remains unclear because it is not known whether they refer to a mole of DNA molecules or to a mole of "segments" or intramolecular regions of DNA molecules. In other words the unit step by which the denaturation process proceeds is not known. The failure of sedimentation studies to settle this point is indeed unfortunate. However, by comparison with other hydrogen bonded systems it appears that the energy of activation and the heat of reaction per base pair should fall within the range of 1 to 10 kcal. Since there are of the order of 10,000 base pairs in a DNA molecule this would imply that values of 10,000 to 100,000 kcal. would be expected if the entire molecule passed from the native state through the transition state into the completely denatured state in one coördinate step. Hence it appears that the unit process cannot involve the entire molecule but only a segment of it composed of 10 to 100 base pairs.

The arguments that can be raised against this

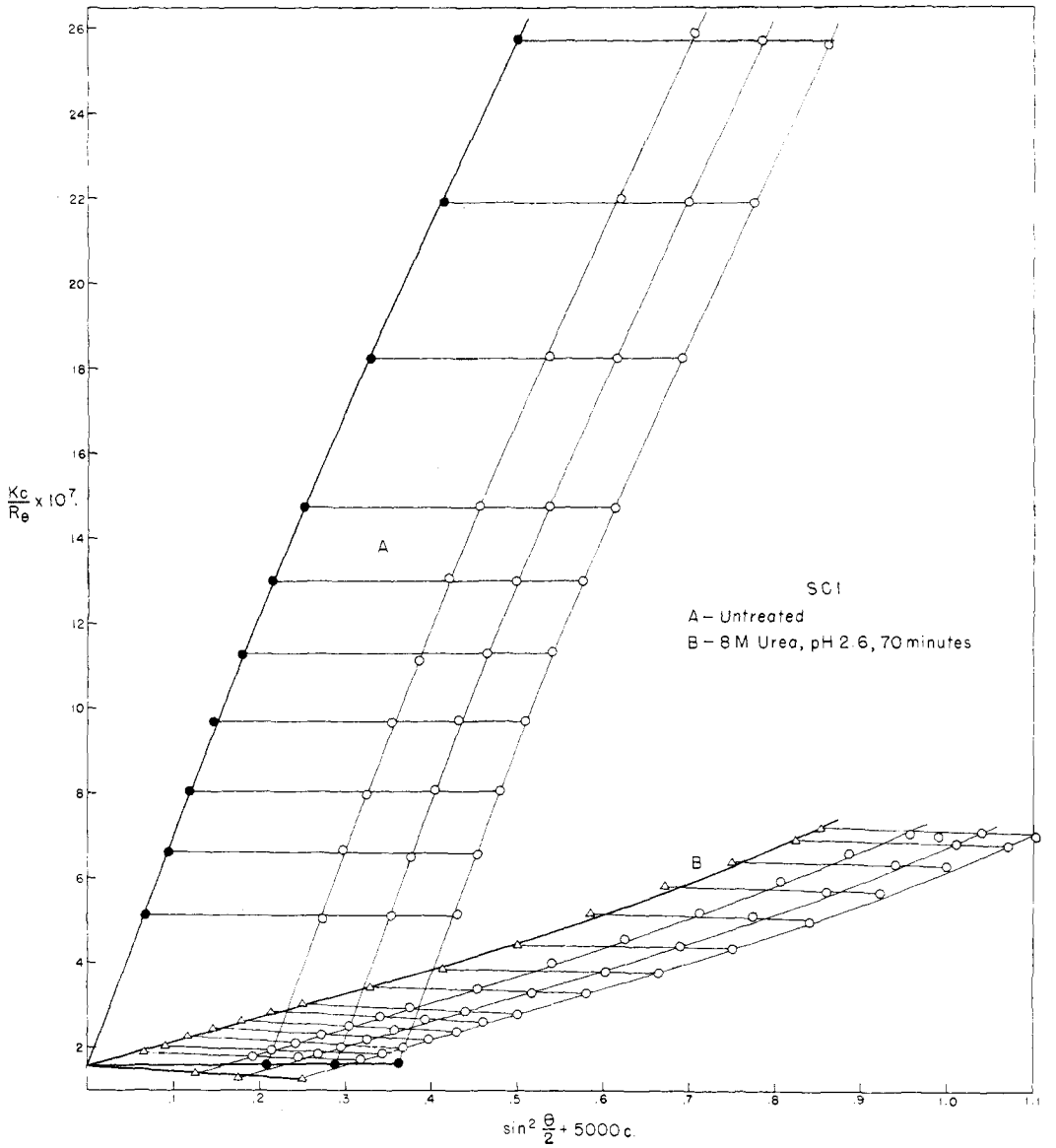

Fig. 10.—Angular distribution of scattered light of sample SC-1 before and after treatment with 8 *M* urea at *p*H 2.6.

interpretation are, however, very serious indeed. Two illustrations will suffice. If the denaturation proceeds by the melting out of one sequence of base pairs after another, the viscosity would be expected to fall sharply when the first few sequences melt out and then respond more gradually. Thus it would not represent a quantitative measure of the extent of the denaturation reaction as assumed in the above calculations. In addition, the previously mentioned difficulties in interpreting the intrinsic viscosity of the denatured state must also extend to the partially denatured state.[20] A more serious difficulty lies in the assumption that equilibrium is achieved at a given temperature and that the distribution so produced is preserved upon cooling. Obviously, much work will have to be done at the temperature of interest in order to assay directly the extent of denaturation and to prove or disprove this assumption.

Thus far no mention has been made of the possible effect of chemical heterogeneity, and indeed the present observations do not require its consideration. Since there are two types of base pairs and since these probably have a difference in stability, the compositional variations revealed by recent fractionation studies[21] would imply variations in the stability of different molecules and it may be expected that this variation would be exhibited among the sequences of segments which may be the unit of the denaturation process. If different regions of the DNA molecules had different ther-

(20) Another indication of the difficulty in interpreting the partially denatured state is shown by comparing the intrinsic viscosity and radius of gyration of material denatured at 94° (Figs. 1 and 4). In this case the radius of gyration has fallen to within 25% of the value for completely denatured DNA while the viscosity has only decreased half-way. Since the radius of gyration depends upon a higher average than the intrinsic viscosity this behavior is obviously incompatible with any simple model of the denaturation process based upon analogies with ordinary polymer molecules.

(21) See, for example, E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. I, Academic Press, New York, N. Y., 1955, p. 358.

mal stabilities depending on their composition, this would, of course, have serious effects on the calculations given above.

Since at this stage we are unable to deduce the mechanism of the denaturation reaction, we would propose the following scheme as a tentative model for which various experimental tests can be designed. At a given temperature above a critical value, certain sequences of nucleotides will be unstable and will melt out quickly. An equilibrium extent of denaturation is soon reached because the denatured regions are bounded by regions having a higher concentration of the more stable base pair. Moreover, as a result of the over-all compositional heterogeneity, denaturation will proceed further in some molecules than in others. Upon raising the temperature to successively higher temperatures more of the undenatured sequence will denature and at a sufficiently high temperature (which may indeed be slightly above that reached in this study) denaturation will be completed. If the temperature is lowered from one of the intermediate temperatures some reformation of the native structure may occur but the longer denatured regions can be expected to remain in this state because of (1) the configurational entropy gain of the separated polynucleotide strands and (2) the formation of irregular hydrogen bonds between non-opposite segments of the two strands. This proposal accommodates most of the observations reported here as well as most of the observations made on the inactivation of transforming principle[22] by means of heat and denaturing agents.

The Dissociation of DNA.—It has been our uniform experience that thermal denaturation alone produces no significant change in the molecular weight *as measured by light scattering.* As previously mentioned, this apparent constancy in molecular weight is indeed real only if the molecular weight distribution in the DNA samples is roughly approximated by the most probable distribution. Considerable indirect evidence does support this assumption but direct proof awaits the actual determination of the distribution. In this discussion this assumption is made.

At the ionic strength employed in this work an exposure of about 15 min. at 100° represents the most severe thermal treatment possible without inducing a gradual and continuous decay in molecular weight. In terms of light scattering, viscosity, titratability of basic groups[3] and the change in optical density at 259 mμ[2] the denaturation reaction has reached completion during this treatment. If this conclusion is strictly true, it would be expected that the two polynucleotide strands making up the DNA molecule according to the Watson–Crick structure would separate and the molecular weight would be reduced by half. Upon finding that this does not occur, we are led to conclude (1) that indeed not every hydrogen bond has been dissociated by this condition, (2) that the potential hydrogen bonding capacity of the polynucleotide chains is such that some hydrogen bonds form again between the two previously mated chains or (3) that the time required for the disentanglement of the two intertwined strands is longer than the period of time in which the DNA may be exposed to the elevated temperature without risking chemical degradation. Of course, the possibility must be allowed that the strands are united by very infrequently occurring chemical bonds that have escaped identification.

Each of these points deserves brief comment. The sensitivity of the means available for estimating the extent of denaturation is such that several hundred bonds could remain intact in each molecule without being observed. Moreover, under these conditions the denaturation takes place in a temperature range adjacent to 100° and as a consequence the possibility of safely exceeding the denaturation range is excluded. In addition, the argument can be made that the driving force for the dissociation of the last hydrogen bonds may be greatly diminished because by this stage the gain in configurational entropy may have reached its maximum value. Then the driving force would be only the gain in translational entropy that would result from dissociation of the two strands. Using the Sackur–Tetrode equation for a crude estimate, one finds at a concentration of 50 mg./dl. an entropy change of about 100 e.u. and this in turn requires an energy of binding of the two strands of about 30 kcal. in order to prevent dissociation. From this it appears that a small number of hydrogen bonds (of the order of 3 to 30) would be sufficient to prevent dissociation. Thus the coöperative nature of the denaturation can remain as previously envisioned, but if as few as several hydrogen bonds per thousand remained, the dissociation of DNA would not occur.

The second possibility mentioned above, the formation of other hydrogen bonds between denatured strands, is supported by the observation that aggregation and indeed gel-formation occurs following thermal denaturation at higher concentrations. The proximity of the two previously mated polynucleotide strands during and following denaturation ensures that hydrogen bonds will form if this is possible. From the arguments of the previous paragraph, only a few would be needed to keep the two strands together. At this point, it is relevant to emphasize that fairly extensive hydrogen bonding between the previously mated chains could develop when the temperature is lowered without contradicting any of the previously mentioned evidence. That is, if the newly formed bonds did not unite the previously matched base pairs but rather only led to a disorganized and incomplete pairing, the titratability of the groups involved would remain normal. Moreover, the observed 15% rise in optical density (measured at room temperature following thermal denaturation) is far short of the maximum possible, that is, the 55% attained in complete hydrolysis.[2] Consequently, the possibility of substantial hydrogen bonding of a disorganized type in the denatured material at room temperature appears possible. A structure of this kind would resemble a highly swollen gel particle and may offer a means of explaining the curious hydrodynamic behavior exhibited by denatured DNA.

The final possibility, the requirement of a long

(22) See, for example, S. Zamenhof in "Progress in Biophysics and Biophysical Chemistry," Vol. 6, Pergamon Press, London, 1956, p. 85.

period of time for disentanglement, has a picturesque appeal. However, the time scale in which equally long polymer chains of rubber-like material explore a wide range of configurations, as exhibited in the fast retraction of stretched rubber, argues strongly against this view. Again, however, the existence of transient hydrogen bonds, forming and dissociating in such a way as to greatly prolong the time required for diffusional movements of the polynucleotide strands, could perhaps extend the time scale enough to account for a delay of the order of minutes.

With these three different possibilities, each capable of explaining the absence of dissociation in DNA under the conditions described, the failure to observe the dissociation is not unexpected. Nevertheless, it would be desirable to bring about the complete dissociation if possible. It would, for example, provide polydesoxyribonucleotide material for experimentation, permit through molecular weight measurement the direct assessment of the number of interruptions, if any, that may occur in the individual polynucleotide strands and it would add belatedly additional evidence for the existence of the Watson–Crick model in solution.

With this in mind, we have made several attempts to bring about the dissociation of DNA by carrying out both acid and thermal denaturation in the presence of 8 *M* urea. In a typical case, the DNA solution in saline–citrate which had been made 8 *M* in urea was brought to *p*H 2.6 for about 1 hour. Then the urea was removed by dialysis against saline–citrate solvent. The light scattering results on this sample compared with the untreated DNA (sample SC-1) are shown in Fig. 10. It is clear that there is no change in the light scattering molecular weight but there is a contraction to the much smaller molecular size; the radius of gyration changes from 2600 to 950 Å.; the value characteristic of the completely denatured state.

Repetition of this and some related experiments, some of which are summarized in Table II, have failed to produce a drop to one-half in molecular weight unless degrading conditions were maintained for the right length of time (see *p*H 2.4 entry in table). However, there are so many possibilities to explore that eventual success in obtaining dissociation without degradation is by no means unlikely. However, it appears appropriate to note here our doubts concerning the claim of Alexander and Stacey[23,24] to have achieved this dissociation. They have reported that a fall to one-half of the original molecular weight could be brought about by (1) exposure to *p*H 2.2 followed by neutralization and (2) exposure to 4 *M* urea at room temperature. In the latter case, a previous treatment of the DNA with ethylenediamine tetraacetate was found to be necessary for thymus DNA but not for herring sperm DNA.

(23) P. Alexander and K. A. Stacey, *Biochem. J.*, **60**, 194 (1955).
(24) P. Alexander and K. A. Stacey, *Nature*, **176**, 162 (1955).

TABLE II

SUMMARY OF ATTEMPTS TO DISSOCIATE DNA

Sample	Condition	Time of exposure, min.	After removal of urea M_w	ρ_g, Å.
V-1	8 *M* urea, *p*H 2.7	150	8,100,000	1200
	8 *M* urea, *p*H 2.6	45	8,400,000	1120
	8 *M* urea, *p*H 2.4	30	4,100,000	1060
SC-1	8 *M* urea, *p*H 2.6	70	6,700,000	950
	8 *M* urea, *p*H 2.6	70	6,700,000	950

In a recent report from this Laboratory[4] it has been shown that chemical degradation can be detected at *p*H 2.6 and that this increases rapidly with further lowering thereof. The degradation is continuous showing no tendency to stop at half the original value. Hence we consider the above-mentioned observations to be fortuitous.

With regard to the claim that dissociation occurs in 4 *M* urea we can only suggest that during some part of the operation of adding and removing the urea, salt may not have been present. Under these conditions, as mentioned in the earlier discussion of Conway's work, denaturation may have occurred and been followed by dissociation. However, the implication in the reports is that salt was continuously present. With salt present at room temperature even denaturation, a necessary prerequisite to dissociation, does not occur. This point was checked in particular with herring sperm DNA samples kindly provided by Dr. Alexander. In this case, the intrinsic viscosity of the DNA solution was unchanged after exposure to 4 *M* urea in saline–citrate solvent. Moreover, the urea treated sample showed the characteristic 15% increase in optical density at 259 mμ and the 12-fold fall in intrinsic viscosity after heating to 100°. This also showed that the native structure was intact after the exposure to urea. Similar results were obtained with thymus DNA before and after treatment with ethylenediamine tetraacetate. It is on this basis that the present evidence for the dissociation of DNA seems unconvincing.

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