Kinetic Studies of Denaturation and Reaction with Formaldehyde on Polydeoxyribonucleotides*

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ABSTRACT: Kinetic experiments on the reaction between formaldehyde and double-helical DNA such as poly(dA·dT), poly(dA-T), and *Escherichia coli* phage T7 DNA have been carried out using the spectrophotometric method. The reaction mixture contained 0.04 M sodium borate–1.7 mM boric acid, pH 9.1, 3.7% (by weight) formaldehyde, and about 20 μg/ml of DNA. By the hyperchromicity measurements of ultraviolet absorption at two specific wavelengths we could estimate separately the extent of reaction, χ_r, and the extent of denaturation, χ_d. The kinetic experimental results can be explained quantitatively by a theory in which the frequent opening and closing or "breathing" of base pairs and the spontaneous permanent opening of bases adjacent to a reacted base pair are the two important elementary processes.

The experimental parameters of the breathing and of the induced denaturation of poly(dA-T) and poly(dA·dT) are greater than those of T7 DNA suggesting the presence of defects in the duplex DNA structure. These parameters show a sharp decrease in a certain temperature range similar to the increase in ultraviolet absorption in the thermal helix–coil transition of DNA.

The static aspects of the physicochemical properties of the double-helical structure of deoxyribonucleic acid have been extensively studied by optical, hydrodynamical, and many other methods. The recent work of Printz and von Hippel has focused interest on the dynamic aspects of the double-helical structure (Printz and von Hippel, 1965; von Hippel and Printz, 1965). They have shown, using the hydrogen exchange method developed by Englander (1963), that DNA is subject to local structural fluctuations which result in frequent opening and closing or "breathing" of base pairs. This idea is obviously important in the mechanism of DNA unwinding and the related problems such as DNA replication. While the hydrogen exchange method is quite useful for the study of the dynamic properties of the DNA helix inasmuch as this method does not perturb the structure itself, the temperature of the reaction is limited to quite low temperatures.

In this paper we demonstrate that kinetic studies on the reaction between the double-helical DNA and formaldehyde may provide another approach to the dynamic properties of DNA helix.

Like the hydrogen exchange, the formylation of bases in duplex DNA molecules will not occur unless the base pairs are opened. Therefore, the fraction of the total time that a base pair is separated may be estimated from the ratio of the rate of reaction on double-helical DNA to the rate of reaction on single-stranded DNA. Furthermore, the kinetic studies of the reaction will be useful for the quantitative investigation of defects in double-helical DNA such as single-stranded ends in λDNA, interruptions in polynucleotide chains in bacteriophage T5 DNA, and the hairpin structure in poly(dA-T). There is another important characteristic of the reaction which may provide new information of the duplex DNA structure. That is, DNA bases are made incapable of forming the base pairing once they are reacted. For example, since a number of base pairs may be separated simultaneously in a cooperative fashion, a reacted base pair will induce the permanent separation of adjacent base pairs. The extent of this induction effect may depend on the number of base pairs breathing cooperatively as a unit, and hence on the duplex DNA structure.

For these purposes, the fraction of base pairs reacted with formaldehyde, the fraction of base pairs denatured but unreacted, and the fraction of base pairs in the native double-helical structure have to be obtained as a function of reaction time. Ultraviolet absorption measurements at a wavelength, where the extinction coefficient of a single-stranded DNA coincides with that of reacted DNA, have been used to estimate the fraction of base pairs in the native double-helical structure. The simultaneous measurements of ultraviolet absorption at another wavelength, where the fractional increase in the optical density upon the reaction of double-helical DNA exhibits a maximum, have been used to obtain the fraction of reacted base pairs.

Bacteriophage T7 DNA had been used to investigate the breathing in a duplex DNA without any defects in the base pairing. Poly(dA-T) and poly(dA·dT) have been examined so that by comparison with the results for T7 DNA the effect of defects in duplex molecules may be observed. Poly(dA-T) and poly(dA·dT) have also been used to investigate the temperature dependence of the breathing, and the effects of duplex ends have been examined by using poly(dA-T) samples of various molecular weights.

A theory has been developed to estimate from the kinetic experimental results useful parameters of the reaction and to consider the results in terms of the structure of duplex DNA molecules.

Experimental Section

Materials and Methods

Poly(dA-T) and poly(dA·dT) were provided by Dr. Patrick Cassidy. Poly(dA-T) samples of various molecular

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weights were prepared by hydrolyzing the original sample with DNase. Pancreatic DNase solution of concentration 0.080 μg/ml contained 1 mg/ml of bovine serum albumin, 7 mM $MgCl_2$, and 0.05 M Tris (pH 7.5). To 6.5 ml of poly(dA-T) solution of concentration 140 μg/ml, which had been dialyzed *vs.* 0.001 M Tris–0.5 M NaCl and preheated to 37° for 10 min, 50 μl of DNase solution was added and incubated. At various times after the addition of DNase solution, an aliquot was separated, heated at about 80° for 10–15 min to stop the reaction, and dialyzed first against 0.01 M Tris (pH 7.4)–0.001 M EDTA, and then against a standard buffer solution containing 0.04 M sodium borate–1.7 mM boric acid (pH 9.1).[1]

The crude poly(dA·dT) sample was purified by the preparative alkaline CsCl density gradient centrifugation to separate the contaminated poly(dA-T) and to mix the poly(dA) and poly(dT) components exactly in one to one molar base ratio. A solution for centrifugation contained 0.02 M NaOH, 5.7 M CsCl, and about 30 OD_{260} units of the crude polymer. It was centrifuged at 25° in a Spinco Model L centrifuge using a Type 40 rotor at 39,000 rpm for 48 hr. A few drops of mineral oil was layered on the top of each tube to stabilize the gradient. Fractions (*ca.* 0.10 ml) were diluted with 0.2 ml of 0.05 M Tris (pH 7.5) and the optical density was read in a 1-mm path-length cell. The appropriate fractions were dialyzed extensively against 0.01 M Tris (pH 7.4)–0.001 M EDTA. The solutions of poly(dA) and poly(dT) were then mixed exactly in one to one molar base ratio. The ratio of the extinction coefficients, $\epsilon_{dT}/\epsilon_{dA}$, at 260 m$\mu$ was 1.01 (values ranging from 0.89 to 1.13 were reported by Chamberlin (1965)). The mixture was made 0.05 M NaCl, heated to 70°, and cooled gradually to room temperature. The final dialysis was made overnight against the standard buffer solution. The heating treatment was necessary to obtain a sharp symmetric band in the analytical CsCl density gradient.

E. coli phage T7 DNA was prepared in the following way. The bacteria *E. coli* B was grown in the glycerol medium in a 4-l. flask under shaking up to an OD_{650} of 0.4 (7×10^8 bacteria/ml). After the addition of the phage stock solution incubation was continued until OD_{650} decreased to as low as 0.05. The incubation was stopped by chilling in ice and the solution was filtered through a Celite layer. The phage was sedimented and the pellet was layered on the suspension medium (0.01% gelatin, 1 mM $MgCl_2$, 0.1 M NaCl, and 0.01 M Tris, pH 8.4), left overnight in the cold, and resuspended by the gentle shaking. The differential centrifugation was repeated and the concentrated phage suspension was then subjected to preparative CsCl density gradient centrifugation. The phage layer was collected and dialyzed for 24 hr against the extraction medium (0.9 M NaCl, 0.01 M Tris (pH 7.5), and 0.02 M EDTA). DNA was then extracted by shaking three times with phenol which had been distilled over argon and saturated with 0.01 M Tris buffer solution (pH 7.5).

Sedimentation coefficients were measured using a Beckman-Spinco Model E ultracentrifuge either by the usual moving-boundary sedimentation or by band sedimentation (Vinograd *et al.*, 1963; Studier, 1965). In the moving-boundary sedimentation a 30-mm path-length cell was used to reduce the sample concentration.

The melting temperature, T_m, was determined as usual by measuring the optical density at 260 mμ. A cell housing attached to a Beckman DU spectrophotometer was heated by circulating ethylene glycol aqueous solution whose temperature was increased at a rate 0.16 deg/min. The temperature of the test solution in the cell was measured by using a thermistor which is immersed in mineral oil layered on the top of the solution. A X-Y recorder was used for the automatic recording of the optical density and the temperature.

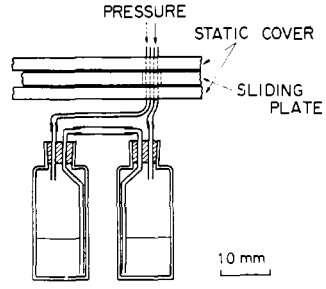

FIGURE 1: The schematic representation of the apparatus for the rapid mixing of a DNA solution and a formaldehyde solution contained separately in two cuvets in the thermostated cell housing of the spectrophotometer. The cuvets are the ordinary 1-cm path-length cells with ground-glass stoppers. The pressure was applied alternately through the two tubings led outside of the housing to ensure the thorough mixing.

Reagent grade 37% formaldehyde (Merck) was used for all experiments without any further purification. However, it was pretreated according to the procedure described by Freifelder and Davison (1963). The final reaction mixture contained 3.7% (by weight) HCHO, 0.04 M sodium borate, 1.7 mM boric acid, and 20 $\sim$ 30 μg/ml of polynucleotides.

The change in optical density with time was measured in a Gilford Type 2000 automatic recording spectrophotometer equipped with a constant-temperature mantle. Each time the optical density of a solution was recorded on the chart, the blank value was also recorded in order to minimize the error due to the noise or the fluctuation of the intensity of the light source. The frequency of the measurement and the speed of the chart could be controlled, the fastest speeds being once in every 12 sec and 2 in. per min, respectively. Expansion of the scale on the chart for only the change in optical density of solutions was possible by shifting the base line for solutions only and adjusting the full scale to the total increase in the optical density. The measurements at two wavelengths, λ_0 and λ_m,[2] were made in two separate experiments under otherwise the same conditions. The temperature of the solution in the cell could be maintained constant within $\pm 0.1°$. Since the rate of reaction of formaldehyde with polynucleotides is quite sensitive to the temperature, the formaldehyde and polynucleotide solutions were separately brought to temperature equilibrium before mixing. For this purpose, the polynucleotide solution and the formaldehyde solution were first put into two separate quartz cells of ordinary 1-cm path length. The two cells were then connected as shown in Figure 1 by small glass tubings and Teflon tubings so that the liquid in the two cells could be mixed rapidly by applying slight pressure from outside after they were put into the cell housing and brought to the temperature equilibrium.

[1] This is used as the standard buffer solution throughout unless otherwise indicated.

[2] The wavelength of light for which the extinction coefficient of denatured but unreacted DNA coincides with that of reacted DNA is designated as λ_0 and the wavelength of light for which the hyperchromicity of reacted DNA exhibits a peak as λ_m. The hyperchromicity is defined here as the fractional increase in the extinction coefficient over the value for native DNA.

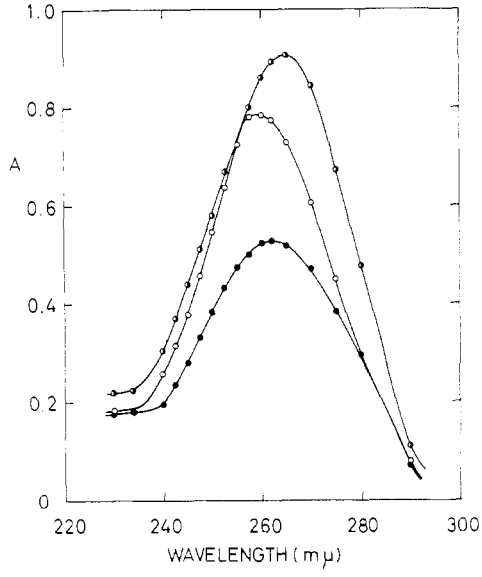

FIGURE 2: Ultraviolet absorption spectra of native (●), denatured (○), and reacted (◑) samples of poly(dA-T) at 42.5°. The optical densities of a poly(dA-T) sample were measured in 0.04 M sodium borate–1.7 × 10^{-3} M boric acid (pH 9.1) at 30.2° and the results were corrected for the temperature difference to obtain the spectra for the native form at 42.5°. The same poly(dA-T) solution was heated to 62.6° to denature the sample and the optical densities were measured. The data were converted into that at 42.5° referring to the data of the temperature dependence of the optical density of the denatured form which was obtained on poly(dA-T) in a very dilute salt solution, 1.5 × 10^{-3} M sodium borate. The spectrum for the reacted poly(dA-T) sample was measured at 42.5° on a poly(dA-T) solution which was prepared by making the above poly(dA-T) solution to 3.7% HCHO by weight and incubating at 70° to the constant optical density. The DNA concentrations for the three spectra are the same.

Estimates of the Extent of Reaction and the Extent of Denaturation from Spectrophotometric Measurements. The increase in ultraviolet absorption upon denaturation of double-helical polynucleotides is well known as hyperchromism. When denatured polynucleotides are further reacted with formaldehyde, the peak absorption increases and its position shifts slightly to a longer wavelength. The absorption spectra of various forms of poly(dA-T) are shown in Figure 2, as an example. It is evident from this that at a certain wavelength, λ_0, the extinction coefficient of denatured but unreacted DNA coincides with that of reacted DNA. Therefore the measurements of ultraviolet absorption at this wavelength can be used to provide the fraction of base pairs in the native form,[3] χ_n, at time, t, as follows.[4]

$$\chi_n(t) = 1 - \frac{H(\lambda_0,t)}{H_0(\lambda_0)} \tag{1}$$

[3] The fraction of base pairs in the native form is designated as χ_n and that of reacted base pairs as χ_r. The fraction of base pairs in the denatured form but unreacted with formaldehyde is designated as χ_d. The fraction of base pairs that are not hydrogen-bonded is the sum of χ_r and χ_d.

[4] $H_m(\lambda_m)$ and $H_0(\lambda_m)$ represent the hyperchromicity due to complete reaction and that due to complete denaturation at the wavelength, λ_m, respectively and $H_0(\lambda_0)$ represents the hyperchromicity due either to complete denaturation or to complete reaction at the wavelength λ_0. $H(\lambda_m,t)$ and $H(\lambda_0,t)$ represent the hyperchromicity at time, t, for the wavelengths λ_m and λ_0, respectively.

On the other hand, the extent of reaction, χ_r, at time, t, may be estimated from the measurements of ultraviolet absorption at the wavelength λ_m, where the hyperchromicity due to reaction exhibits a peak, provided that the contribution due to the denaturation is properly corrected for. It can be easily shown that χ_r is given by eq 2

$$\chi_r(t) = \frac{H(\lambda_m,t) - \kappa H(\lambda_0,t)}{H_m(\lambda_m) - H_0(\lambda_m)} \tag{2}$$

where κ is defined by

$$\kappa = \frac{H_0(\lambda_m)}{H_0(\lambda_0)} \tag{3}$$

Finally, the extent of base pairs in the denatured form at t can be obtained from the normalizing condition

$$\chi_d(t) = 1 - \chi_n(t) - \chi_r(t) \tag{4}$$

It will be worth mentioning here that the present analysis assumes no great deviation from microscopic heterogeneity of base composition.

Results

Characterization of the Samples. Molecular weights of all the samples were estimated from the limiting sedimentation coefficients $s^0_{20,w}$ determined in 1.0 M NaCl using the relation presented by Studier (1965). The molecular weights of the poly(dA-T) samples were found to be 0.34 × 10^6 (treated with DNase for 30 min), 0.87 × 10^6 (20 min), 1.45 × 10^6 (10 min), 1.64 × 10^6 (5 min), 2.8 × 10^6 (untreated). The reciprocal of these molecular weights plotted against the time of DNase treatment is linear. The buoyant density of poly(dA·dT) in CsCl was found to be 1.684 at 25°, and the molecular weight was estimated as 6.6 × 10^6. The sedimentation boundaries of the native and alkaline denatured T7 DNA were both extremely sharp and the sedimentation coefficients were in exact agreement with the figures reported by Studier (1965). These results imply that T7 DNA molecules are intact.

The melting curves of poly(dA-T) and poly(dA·dT) were very sharp and T_m's were 56.6 and 63.0°, respectively, in the standard buffer solution. The T_m value for phage T7 DNA was 78.8°, which is in good agreement with the value estimated using the base composition according to the relation of Schildkraut and Lifson (1965).

In order to find the characteristic wavelengths, λ_0 and λ_m, and to estimate the necessary numerical parameters such as $H_0(\lambda_0)$, $H_0(\lambda_m)$, and $H_m(\lambda_m)$, it is required to measure the ultraviolet absorption spectra of native, denatured, and reacted DNA at various temperatures below T_m in the buffer solution containing 0.04 M sodium borate, 1.7 mM boric acid, and 3.7% formaldehyde. The spectra for the native DNA were obtained without adding formaldehyde.

Ultraviolet absorption spectra of denatured DNA cannot be measured at temperatures below T_m and/or in the presence of formaldehyde. It is assumed, therefore, that the spectra in the presence and absence of formaldehyde are the same, and that the temperature dependence of ultraviolet absorption of DNA in the standard buffer solution and that in a very dilute buffer solution (1.5 mM sodium borate buffer) are the same. The melting temperatures of DNA samples in 1.5 mM sodium borate buffer were all below the room temperature

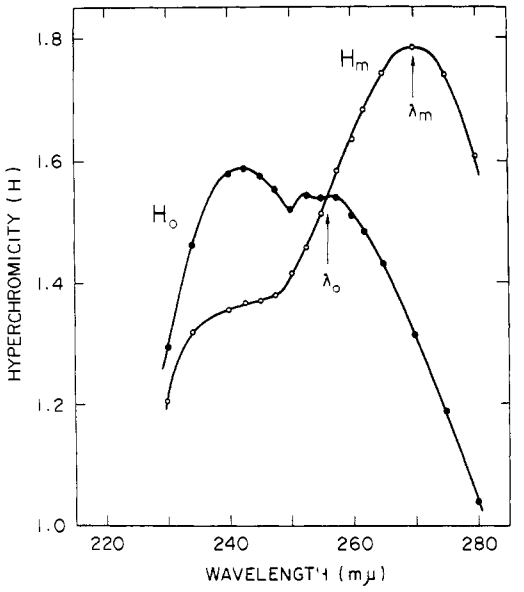

FIGURE 3: The spectra of hyperchromicity, expressed in the fractional increase in the extinction coefficient over the value for native DNA, on poly(dA-T) at 42.5°. The hyperchromicity of denatured DNA and that of formylated DNA are expressed by filled and open circles, respectively.

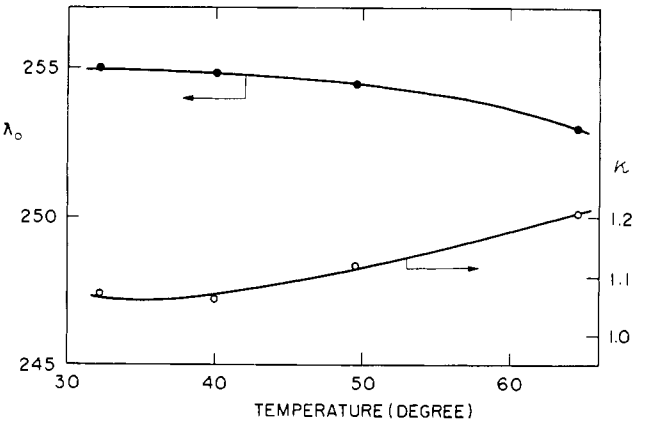

FIGURE 4: The temperature dependence on poly(dA·dT) of the characteristic wavelength λ_0, at which the extinction coefficients of denatured DNA coincides with that of denatured and formylated DNA, and of the ratio of the hyperchromicity of denatured DNA at the wavelength λ_m to that at λ_0. The wavelength λ_m designates the peak position in the hyperchromicity spectrum of formylated DNA.

in agreement with the data of Schildkraut and Lifson (1965). Accordingly, DNA samples dissolved in the standard buffer solution were kept at a temperature above T_m and the ultraviolet absorption spectra were measured. The spectra were converted into those at various temperatures below T_m referring to the temperature dependences of the spectra of DNA samples in 1.5 mM sodium borate.

The ultraviolet absorption spectra of DNA reacted with formaldehyde were measured in a straightforward way. DNA solutions containing 0.04 M sodium borate, 1.7 mM boric acid, and 3.7% formaldehyde were kept at 70° until the peak absorption reached a constant value. The solutions were cooled down and the spectra were measured at various temperatures.

The ultraviolet absorption spectra for poly(dA-T) at 42.5° are shown in Figure 2, and the hyperchromicity spectra calculated from these results are shown in Figure 3.

Due to the slight increase in the ultraviolet absorption of denatured polynucleotides with temperature, both the characteristic wavelength λ_0 and the hyperchromicity ratio, κ, depend slightly on temperature. For poly(dA-T) this effect is negligible, but this is not the case for poly(dA·dT).[5] This dependence for poly(dA·dT) is shown in Figure 4.

Kinetic Experimental Results with Denatured Polydeoxyribonucleotides. There are two kinds of reactive sites in nucleic acids possibly susceptible to the formylation reaction. They are the basic amino groups of adenine, guanine, and cytosine, and the acidic imino groups of thymine and guanine. However, since the change in the ultraviolet absorption spectrum due to the reaction of imino groups is very small compared to that due to the reaction of amino groups, the whole reaction may be characterized by a single rate constant. This possibility is best examined by performing kinetic studies of the reaction with denatured DNA.

The rate constant k_{dr}[6] for the reaction of bases of single-stranded polynucleotides with formaldehyde is also important in the analysis of the kinetic data on native DNA. In the standard solution for the reaction (0.04 M sodium borate, 1.7 mM boric acid (pH 9.1), and 3.7% formaldehyde) the temperatures of the helix–coil transition of polynucleotides under consideration are found to be much higher than room temperature, and the rate of reaction may be too fast at such temperature to be measured accurately in the present apparatus. We therefore used 1 mM borate buffer solution (pH 9.1) to decrease the T_m below the room temperature, because the rate constant of the reaction between mononucleotides and formaldehyde is reported little affected by the ionic strength (Grossman *et al.*, 1961). The extent of reaction, χ_r, was estimated from the fractional increase in the optical density measured at the wavelength 270 mμ.

The kinetic experimental results on poly(dA-T) and poly(dA·dT) were found to follow straight lines in the plot of simple first-order reaction with the results of $10^2 \times$ rate constant k_{dr} (min^{-1}): 10.68 at 48.0°, 4.21 at 39.8°, 2.35 at 34.2°, 1.38 at 29.2° for poly(dA-T), and 7.33 at 47.6°, 3.39 at 38.8°, 1.27 at 29.5° for poly(dA·dT). The activation energy estimated from the Arrhenius plot was 23.8 kcal/mole for poly(dA-T) and 18.5 kcal/mole for poly(dA·dT). These results may be compared with the activation energy 16.8 kcal/mole for the deoxymononucleotide such as dAMP, dGMP, and dCMP observed by using a reaction mixture which contained 2.88×10^{-5} M nucleotide, 2.76 g/dl of HCHO, and 0.11 M potassium phosphate (pH 6.8) (Haselkorn and Doty, 1961).

Kinetic Experimental Results with Double-Stranded Polydeoxyribonucleotides. The kinetic experiments of the reaction of poly(dA-T) samples with formaldehyde were carried out

[5] The increase in the optical absorption of the single-stranded poly(dA) is explained in terms of the temperature-dependent stacking of bases which is the direct consequence of the hindered internal bond rotation. See, for example, Poland *et al.* (1966).

[6] The rate constant when a base pair is reacted with formaldehyde is designated as k_{nr} when it is in the native form, and as k_{dr} when it is in the denatured form. The rate constant when a base pair in the native form is transformed into the denatured form by the induction effect of other base pairs in the native form being reacted with formaldehyde at the rate k_{nr} is designated as k_{nd}.

TABLE I: Characteristic Wavelengths and Hyperchromicity Values in 0.04 M Sodium Borate Buffer (pH 9.1) and 3.7% Formaldehyde.

T, Temp (°C)	λ_0 (mμ)	λ_m (mμ)	H_0 (λ_0)	H_0 (λ_m)	H_m $\lambda_{(m)}$	κ
			Poly(dA·dT) (mol wt 6.6 × 10^6)			
29.5	255.1	270	0.414	0.442	0.923	1.065
38.8	254.8	270	0.413	0.440	0.890	1.065
43.5	254.7	270	0.369	0.403	0.803	1.092
			Poly(dA-T) (mol wt 2.80 × 10^6)			
20.0	256.1	270	0.522	0.303	0.802	0.581
34.1	256.1	270	0.539	0.311	0.794	0.577
39.8	256.1	270	0.542	0.314	0.791	0.579
			E. coli T7 DNA (mol wt 26 × 10^6)			
58.0	257.5	280	0.460	0.598	0.916	1.301

in the standard buffer at three temperatures, 20.0, 34.2, and 39.8°, which are all below the melting temperature of poly(dA-T). The characteristic wavelength λ_0 was 256.1 mμ for the three temperatures, but the hyperchromicity ratio κ increased slightly with temperature from 0.573 at 20.0° to 0.579 at 39.8°. The necessary parameters are summarized in Table I.

The optical densities at 256.1 mμ and at 270 mμ changed slowly at 20.0° as shown in Figure 5 for the poly(dA-T) sample of molecular weight 2.8 million. From these experimental data one can calculate χ_n, χ_r, and χ_d using eq 1, 2, and 4; the results are shown in Figure 6. It is important to note that both the reaction and denaturation took place at certain positive rates from the beginning. The fraction of reacted base pairs increased linearly during the period covered by the present experiment, whereas the fraction of base pairs in the native form decreased with a slight downward curvature. Correspondingly an accelerative increase in the fraction of denatured but unreacted base pairs is observed in the figure.

At a higher temperature 34.2°, χ_n decreases quite rapidly, reaching half its original value in less than 8 min as shown in Figure 7. The downward curvature in χ_n observed at 20.0° in the initial period of the reaction persisted at 34.2°, too, although it is not clearly detected in the figure. On the contrary, the reaction proceeded relatively slowly and the fraction of reacted base pairs amounted to only 15% when 50% of base pairs are separated. In other words, the reacted base pairs induced a larger number of other base pairs to denature in the initial period of the reaction. The denatured base pairs reacted in the meantime, and χ_r reached unity after a sufficiently long period of time. Therefore, χ_d reached a maximum at some time (17 min at 34.2°) after the initiation of the reaction. The position of the peak and its magnitude will depend on the rate of reaction of denatured base pairs and the rate of denaturation.

The above tendency becomes more pronounced as the temperature is increased to 39.8° (see Figure 8). Here the fraction of base pairs denatured is seen to increase sharply to about 80% in 1 min, but only 10% of base pairs have reacted in the same period.

These experimental results clearly indicate that a base pair which is hydroxymethylated and loses its hydrogen bonding capability induces the spontaneous separation of base pairs in its neighborhood. This *induction effect* becomes more pronounced at higher temperatures.

To estimate the effect of duplex ends, kinetic studies were

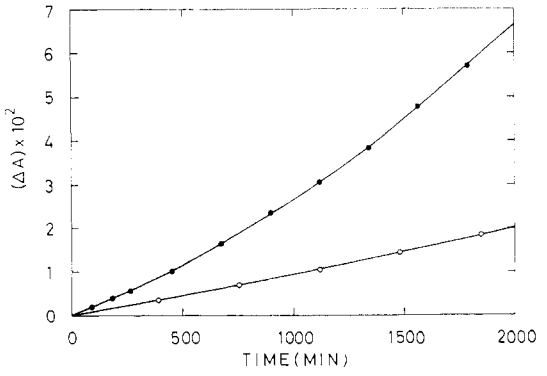

FIGURE 5: The increase in optical density of poly(dA-T) (mol wt 2.8 × 10^6) in the reaction mixture containing 0.04 M sodium borate, 1.7 mM boric acid, and 3.7% formaldehyde at 20.0°. The open circles represent the increments in optical density measured at 270 mμ (η_m) and the closed circles represent those measured at 256.1 mμ (η_0). The optical densities at time zero are 0.124 and 0.437 for η_m and η_0, respectively.

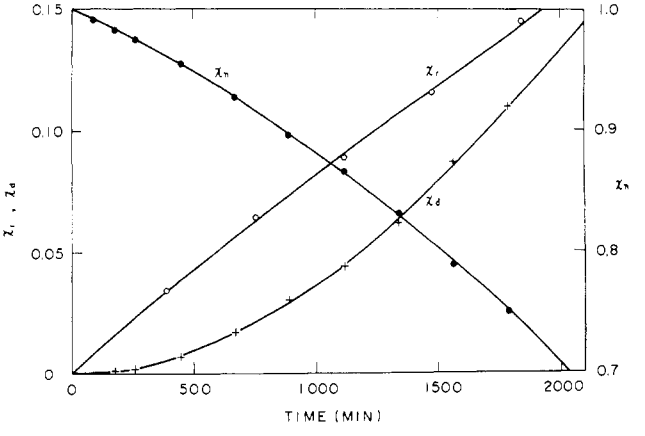

FIGURE 6: Variations with time of χ_n, χ_d, and χ_r on the poly(dA-T) sample of molecular weight 2.8 million at 20.0°. The open circles represent χ_r, the fraction of base pairs that are reacted, and the closed circles represent χ_n, the fraction of base pairs that are in the native form. The fraction of base pairs in the denatured form is shown by crosses. They were calculated from the data represented in Figure 5 using eq 1, 2, and 4.

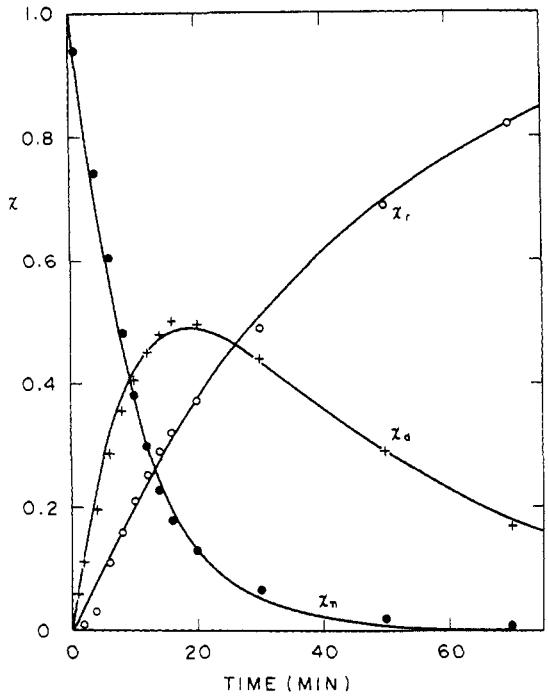

FIGURE 7: Variations with time of three quantities, χ_r (open circles), χ_n (closed circles), and χ_d (crosses), on the poly(dA-T) sample of molecular weight 2.8 million at 34.2°. The solid lines are calculated using eq 16 and 18 with $\mu_1 = 0.101$, $\mu_2 = 0.065$, $\beta = 0.985$, $A_d = -1.02$, and $B_d = -0.0161$.

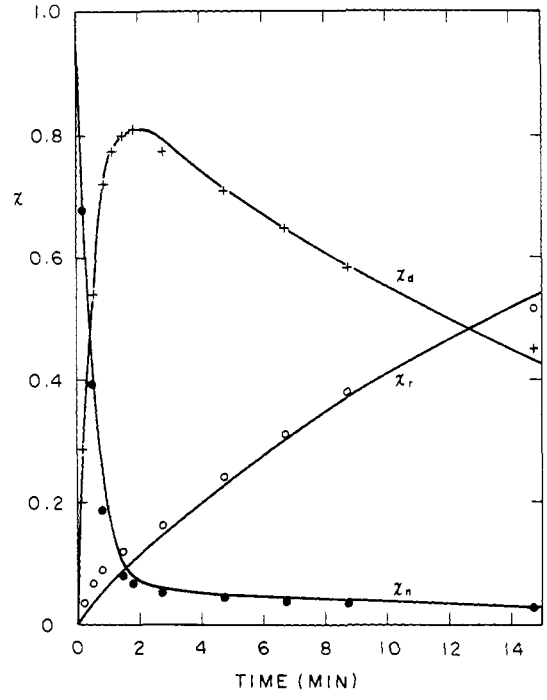

FIGURE 8: Variations with time of three quantities, χ_r (open circles), χ_n (closed circles), and χ_d (crosses), on the poly(dA-T) sample of molecular weight 2.8 million at 39.8°. The solid lines are calculated using eq 16 and 18 with $\mu_1 = 1.96$, $\mu_2 = 0.0426$, $\beta = 0.945$, $A_d = -0.924$, and $B_d = -0.522$.

carried out at 34.2° on the poly(dA-T) samples of different molecular weights prepared by the DNase treatment of the original poly(dA-T) sample. The experimental results on a poly(dA-T) sample of molecular weight 0.34 million are shown in Figure 9 for illustration. On comparing Figure 9 with Figure 7, one can easily note that the initial rate of denaturation is faster and a larger maximum in χ_d appeared at an earlier period of time. However, the feature of the overall time course is similar to the previous data.

As another example, linear double-helical DNA with one kind of base pair of poly(dA·dT) of molecular weight 6.6 million was examined. The reactions were carried out at 29.5, 38,8, and 43.5°. The parameters characterizing the reactions at these temperatures are summarized in Table I. The overall features of changes of the quantities, χ_n, χ_r, and χ_d, with time and how they change with temperature are seen to be quite similar to those on poly(dA-T). The results will be discussed quantitatively in the Discussion section. As a model of linear double-helical DNA with perfectly matched base pairing kinetic experiments on *E. coli* phage T7 DNA were carried out at 58.0°. The characteristic wavelengths λ_0 and λ_m were 257.5 and 280 mμ, respectively, and the hyperchromicity ratio was 1.301. One distinct difference of the results on T7 DNA from those on poly(dA-T) and poly(dA·dT) is that the maximum value of χ_d is very small as shown in Figure 10. This result may imply much smaller induction effect in T7 DNA. However, the quantitative analysis of the kinetic experimental results is necessary to draw this conclusion and to compare the results on *E. coli* T7 DNA to those on poly(dA-T) and poly(dA·dT), because the temperature of the reaction is much higher and the effects of the difference in the molecular weight and the melting temperature among these samples must be considered quantitatively.

Discussion

It has been shown in the previous section that double-helical DNA is transformed into reacted single-stranded DNA in the presence of formaldehyde. The transformation can be followed by measurements of ultraviolet absorptions at two wavelengths, λ_0 and λ_m, which provide us with the fraction of reacted base pairs, χ_r, the fraction of denatured but unreacted base pairs, χ_d, and the fraction of base pairs in the native form, χ_n. If we analyze these experimental data in terms of appropriate kinetic equations, we can expect to derive characteristic parameters that describe

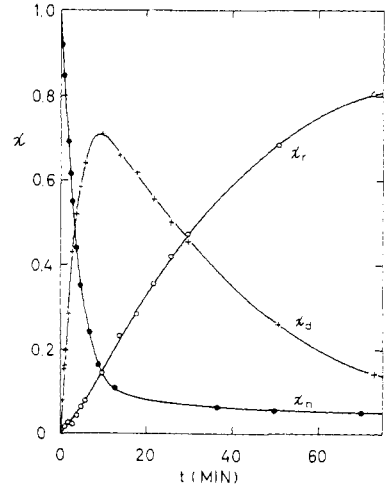

FIGURE 9: Variations with time of three quantities, χ_r (open circles), χ_n (closed circles), and χ_d (crosses), on the poly(dA-T) sample of molecular weight 0.34 million at 34.2°.

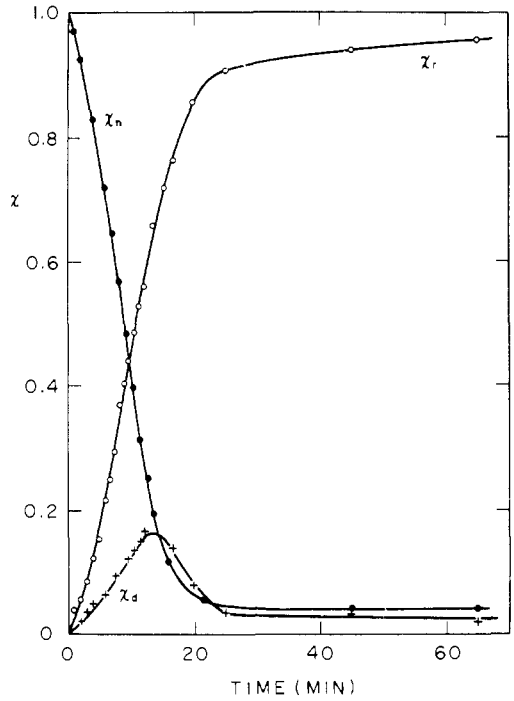

FIGURE 10: Variations with time of three quantities, χ_r (open circles), χ_n (closed circles), and χ_d (crosses), on *E. coli* phage T7 DNA. The reaction temperature is 58.0°.

various properties of the double-helical structure of DNA. For the unambiguous discussion of the mechanism of the transformation, however, it is first necessary to consider the chemistry of the reaction of bases with formaldehyde.

Chemical Reaction of Nucleic Acids with Formaldehyde. Formaldehyde reacts with polydeoxyribonucleotides at various sites of bases. They are basic amino groups of adenine, cytosine, and guanine, and acidic imino groups of thymine and guanine. The involvement of the amino groups has been understood since Fraenkel-Conrat (1954) first pointed it out on the basis of the spectrophotometric studies on DNA, RNA, nucleosides, and nucleotides. It is only recently, however, that the acidic imino groups were also shown to be involved in the reaction, at least in the alkaline pH range (Lewin, 1966). With respect to the types of the products, the evidence increasingly favors hydroxymethylation (Grossman *et al.*, 1961; Michelson and Grunberg-Manago, 1964; Lewin, 1966) over the formation of the Schiff bases, of the type $RN{=}CH_2$ (Fraenkel-Conrat, 1954). In addition to these reactions it is clear that heat-resistant interstrand cross-links are also formed when double-helical DNA is allowed to react with formaldehyde for a long period of time (Freifelder and Davison, 1963). This cross-linking reaction could be minimized and even eliminated at high pH. For example, a sample treated for 24 hr at 25° in 4% formaldehyde–0.04 M borate buffer (pH 9) showed no cross-linking.

We are able to add at this point some results of nuclear magnetic resonance spectroscopic measurements which may support the hydroxymethylation. The reaction mixture containing 10% (by weight) dCMP, 3.7% formaldehyde, and 0.1 M sodium borate in D_2O was incubated for 12 hr at room temperature and nuclear magnetic resonance absorption was measured using a Varian Type A-60 nuclear magnetic resonance spectrometer. We used dCMP because of its large solubility in water. The absorption of Schiff-base-type compound may be clearly detected because it is expected to appear at around 500 ~ 700 cps downfield (Szymanski and Yelin, 1968), and none of the absorption due to dCMP, formaldehyde, and the hydroxymethylated dCMP appears in this region. The results showed no absorption between 500 and 1000 cps downfield. We therefore conclude that no Schiff-base-type compound forms. In addition to this, the reaction of the acidic imino group with formaldehyde was studied spectrophotometrically using poly(dT). The ultraviolet absorption spectra recorded in a Beckman ultraviolet spectrophotometer Type DKII-A before and after the incubation of poly(dT) with formaldehyde showed a red shift of 4 mμ in the peak position as well as a very slight increase in the peak absorption.

Thus, in the reaction of polydeoxyribonucleotides with formaldehyde several minor types of elementary reactions are involved in addition to the principal one of hydroxymethylation. However, the spectrophotometric studies of the reaction on single-stranded poly(dA-T) and poly(dA·dT) show that the reactions followed quasi-first-order reaction kinetics quite precisely. Therefore, the process in which a hydrogen-bonded base pair reacts with formaldehyde while it is breathing can be characterized by a single rate constant.

Analysis of the Initial Stage of the Reaction. The kinetic results obtained on poly(dA-T), poly(dA·dT), and *E. coli* T7 DNA should be compared in terms of rate constants of well-defined processes. We have, therefore, derived kinetic equations based on reasonable mechanisms and have evaluated the parameters by comparing the overall time courses of χ_r and χ_d with the equations. We first focus our attention on the initial stage of the reaction because the initial rates of increase in χ_r and χ_d are related to only a limited number of elementary processes.

We consider a part of a very long DNA molecule which is double-helical and hydrogen-bonded complementary and assume that a base pair along the chain is not hydrogen bonded all the time but is open for a certain fraction of time, θ. This phenomena is called "breathing" of base pairs (Printz and von Hippel, 1965; von Hippel and Printz, 1965). The quantity θ is expressed in terms of the rate constants, k_1 and k_2, of equilibrium 5, as $k_1/(k_1 + k_2)$. Since a base pair in a

$$\text{closed base pair} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{open base pair} \qquad (5)$$

linear sequence of closed base pairs cannot assume an open configuration due to the difficulty of breaking two stacking interactions, a certain number of consecutive base pairs should be separated simultaneously.

In this model, the initial rate of increase in χ_r, designated as k_{nr}, is given by the product of θ and k_{dr}. Once a base pair is formylated and made incapable of closing the hydrogen bond, it will induce the spontaneous and permanent denaturation of some number of the base pairs in its neighborhood. At the initial stage of the reaction, such openings will occur at discreet positions along the duplex DNA. Therefore, the initial rate of increase in χ_d, designated as k_{nd}, is given by the product of the rate constant k_{nr} and an induction parameter, ξ, which expresses the number of base pairs induced to separate by a formylated base pair.

Base pairs at the ends of linear duplex DNA molecules will be characterized by a larger θ than the base pairs in the middle. Therefore, k_{nr} and θ for samples of lower molecular weights will be greater, and so is k_{nd}. If a linear duplex contain

TABLE II: Kinetic Parameters Estimated from Initial Rates of Denaturation and Reaction.

T, Temp (°C)	$T - T_m$ (°C)	$k_{nd} \times 10^4$ [a] (min^{-1})	$k_{nr} \times 10^4$ (min^{-1})	$k_{dr} \times 10^4$ [a] (min^{-1})	$\theta \times 10^2$ [b]	ξ [b]	$MW \times 10^{-6}$
			Poly(dA-T)				
20.0	−36.6	0.11	0.92	53.3	1.7	0.12	2.80
34.1	−22.5	610	34	251	14	18	
39.8	−17.4	17,000	240	477	50	71	
			Poly(dA-T) (DNase)				
34.1	−22.5	1,200	94	251	37	13	1.64
		1,300	84	251	34	15	1.45
		1,500	103	251	41	15	0.87
		1,600	111	251	44	14	0.34
			Poly(dA·dT)				
29.5	−32.5	0.90	1.46	127	1.2	0.62	6.6
38.8	−24.2	206	51	329	15.5	4.0	
43.5	−19.5	4,560	180	486	37	25.3	
			E. coli Phage T7 DNA				
58.0	−20.0	56	349	3760	9.3	0.161	26

[a] k_{nd} and k_{nr} should be replaced by $k_{nd} + nk_{ld}$ and $k_{nr} + nfk_{ld}$, respectively, when we consider the contribution from ends. [b] The end effects are not considered.

short single-stranded ends as λDNA (Hershey *et al.*, 1963; Ris and Chandler, 1963; MacHattie and Thomas, 1964) and/or interruptions in the polynucleotide chains as T5 DNA (Abelson and Thomas, 1966), these rate constants should further increase. Thus it should be possible to determine the concentration of the defects in the DNA double-helical structure (Triphonov *et al.*, 1967, 1968), but this is not the main purpose of this paper.

The sample of poly(dA·dT) prepared by mixing poly(dA) and poly(dT) may contain both the unpaired bases and interruptions. The poly(dA-T) samples are very special in that the base pairs are formed in the intramolecular fashion. By contrast, T7 DNA is a perfect linear duplex molecule. Therefore, it is interesting to compare the results on poly(dA-T), poly(dA·dT), and T7 DNA.

The rate constant k_{dr} was estimated previously from kinetic experiments in very dilute salt solutions. However, it can also be estimated directly by analyzing the present kinetic data for the late period of the reaction as follows. At an elevated temperature the rate of formylation of open base pairs is small compared to the rate of denaturation as shown in the previous section. Hence, at a late period of the reaction, χ_n decreases to a very small value and its variation with time is small, too. Therefore, the essential transformation of the material among the species is from χ_d to χ_r. If we assume an exponential decrease in χ_n with time as

$$\chi_n = a \exp(-bt) \tag{6}$$

then the material balance for χ_d is expressed by

$$d\chi_d/dt + d\chi_n/dt + k_{nr}\chi_n + k_{dr}\chi_d = 0 \tag{7}$$

Using eq 6 and considering the initial conditions $\chi_n = 1$, $\chi_d = 0$, and $t = 0$, we obtain for the solution

$$\chi_n + \chi_d/\zeta = \exp(-k_{dr}t) \tag{8}$$

where ζ is given by

$$\zeta = (b - k_{nr})/(b - k_{dr}) \tag{9}$$

The rate constant k_{nr} is estimated from the initial increase in χ_r. Therefore, if we plot, against the arbitrary number of ζ, the parameter k_{dr} calculated from eq 9 and that estimated from the experimental values of χ_n and χ_d with the use of eq 8, we obtain k_{dr} from the ζ value at the intersection of the two curves. An example of the analysis of the data on poly(dA-T) at 34.2° is shown in Figure 11 and k_{dr} is estimated as 2.51×10^{-2} min^{-1}. The values of k_{dr} estimated thus are very close to the data obtained previously in very dilute salt solutions. The results thus obtained are summarized in Table II. The rate constants k_{nd} and k_{nr} were estimated from the initial rates of increases of optical densities at the wave-

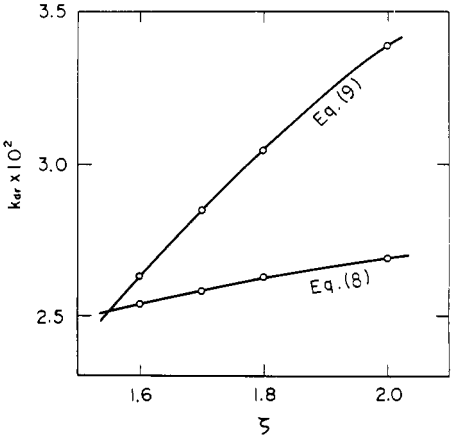

FIGURE 11: The estimate of the rate constant k_{dr} of the reaction between denatured DNA and formaldehyde from the late stage of the reaction of double-stranded DNA with formaldehyde. The experimental data were taken from Figure 6 on poly(dA-T) at 34.2°, which is characterized by $b = 6.1 \times 10^{-2}$ and $k_{nr} = 0.34 \times 10^{-2}$ min^{-1}.

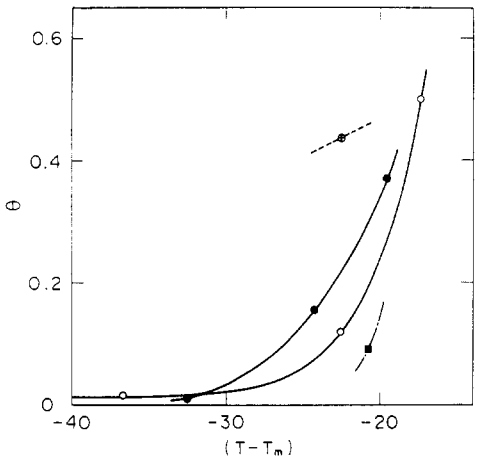

FIGURE 12: The temperature dependence of the breathing parameter θ on poly(dA-T) (○), hydrolyzed poly(dA-T) (⊗), poly(dA·dT) (○), and *E. coli* phage T7 DNA (■). The molecular weights of the samples are given in Table II. T_m represents the temperature of helix–coil transition in the standard buffer solution.[1]

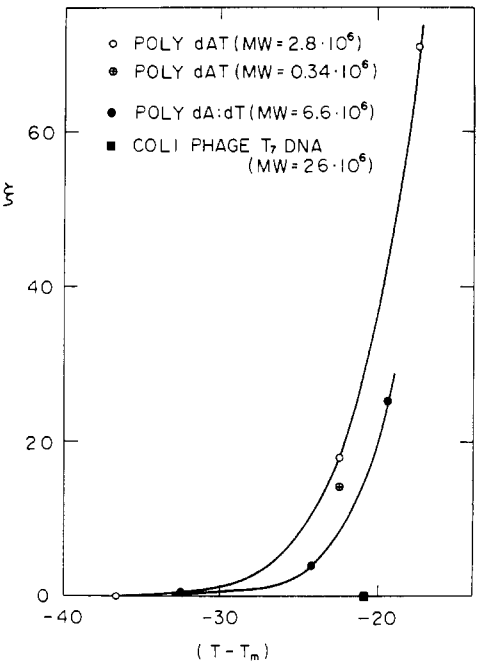

FIGURE 13: The temperature dependence of the induction parameter on poly(dA-T), poly(dA·dT), and *E. coli* phage T7 DNA.

lengths λ_0 and λ_m, which were read directly from the recording chart. The rate constants k_{nd} and k_{nr} so estimated for poly(dA-T) samples of different molecular weights increase with decreasing molecular weight as expected from the end effects. Therefore, the end effects should be corrected to obtain the rate constants of the reaction due entirely to the breathing. The value of k_{nr} for infinite molecular weight can be obtained by the extrapolation in the plot of k_{nr} *vs.* reciprocal molecular weight, with the result of 80×10^{-4} min^{-1}. This value is compared with 34×10^{-4} min^{-1} for the original poly(dA-T) sample. The difference between these two values may be considered as indicating some difference between these samples in the way the bases are paired in the molecule. In any case, the essential role of breathing of base pairs in the reaction of duplex DNA molecules and formaldehyde can be unambiguously demonstrated, as shown in the next section, by deriving kinetic equations and by comparing them with kinetic results.

Since k_{dr} is constant independent of the molecular weight, the parameter θ is greater for the poly(dA-T) sample of smaller molecular weight. On the contrary, the induction parameter, ξ, seems to be constant. The small values of θ and ξ for T7 DNA clearly indicate the completely matched linear sequence of base pairs in this molecule. In order to compare the results on poly(dA-T), poly(dA·dT), and T7 DNA, the parameters θ and ξ are plotted in Figures 12 and 13, respectively, against $T - T_m$, where T_m is the helix–coil transition temperature in 0.04 M sodium borate buffer (pH 9.1). It is seen for both poly(dA-T) and poly(dA·dT) that the parameter exhibits the steep transition-like increase but it is much less sharp than the increase in the optical density observed on the thermal helix–coil transition. The θ value for T7 DNA is small whereas that for the degraded dAT is quite large, in agreement with the consideration stated previously. It may well be expected that the increase in θ will be less sharp for double-helical molecules with more defects such as single-stranded breaks and ends. If we define the transition temperature at $\theta = 0.5$ as in the thermal melting temperature the presence of formaldehyde decreases T_m by about 17°.

The induction parameter ξ also shows a sharp increase in the same temperature range but the discussion on the parameter θ in terms of the molecular structure seems not to be directly applicable to ξ, because the magnitude of ξ for the degraded poly(dA-T) sample is a little less than that for the original sample. The experimental values for poly(dA·dT) are smaller. But the estimated value for T7 DNA is only 0.16 at the temperature where the parameters for other samples range from 14 to 28. Therefore, although there is no straight parallelism between θ and ξ, they are smallest for the long, completely matched double helix without single-strand breaks.

Theoretical Deviation of Kinetic Equations and the Comparison to Experiment. In the previous section, it has been demonstrated that the effects of single-stranded breaks and ends must be considered quantitatively in order to conclude that the breathing of base pairs is playing an essential role in the reaction between the double-helical DNA and formaldehyde. With this in mind we wish to consider the overall time courses of χ_r and χ_d using an appropriate model. We start again from eq 5, the equilibrium between the bonded and unbonded states for each base pair of an infinitely long molecule. As discussed in the previous section the fraction of total time θ that a base pair in the native DNA molecule is separated and exposed to the reaction with formaldehyde is given in terms of the forward and backward rate constants k_1 and k_2 as $k_1/(k_1 + k_2)$. The rate constant of formylation of native DNA, k_{nr}, is given by the product of θ and k_{dr}. The formylated base pair induces the spontaneous separation of ξ base pairs in the neighborhood, producing an unbonded loop of the size $\xi + 1$. Therefore, k_{nd} is defined as ξk_{nr}. The size of the loop will increase in both directions, at a rate k_{ld}, due to the reaction of bases in the loop or those of the bonded base pairs adjacent to the loop. For example, if one of ξ base pairs adjacent to a loop reacts with formaldehyde, it will not produce a new loop but simply increase the size of the loop. The number of the unbonded loops will increase in the beginning of the reaction at the rate k_{nr}, but will decrease in the meantime due to the mutual fusion of the growing loops. The rate of the fusion of two unbonded loops is assumed to be proportional to the number of unbonded loops in the molecule.

One can write down a set of differential equations for such processes as

$$d\chi_n/dt + (k_{nd} + k_{nr})\chi_n + k_{ld}N = 0 \quad (10a)$$

$$d\chi_d/dt + k_{dr}\chi_d - k_{nd}\chi_n - k_{ld}N = 0 \quad (10b$$

$$d\chi_r/dt = k_{dr}\chi_d - k_{nr}\chi_n = 0 \quad (10c)$$

$$dN/dt - k_{nr}\chi_n + k_{NN}N = 0 \quad (10d)$$

In the above equations, N is the ratio of the number of unbonded loops to the total number of base pairs in a molecule and k_{NN} is the rate constant of the fusion of two loops. Considering the normalization condition, $\chi_n + \chi_d + \chi_r = 1$, only three of the above relations are independent. The solutions for χ_n and N are obtained with use of eq 10a and 10d, and the initial conditions, $\chi_n(t = 0) = 1, N(t = 0) = 0$, as

$$\chi_n = \alpha \exp(-\lambda_1 t) + (1 - \alpha) \exp(-\lambda_2 t) \quad (11)$$

$$N = \{\alpha(1 - \alpha)(\lambda_1 - \lambda_2)/k_{ld}\}\{\exp(-\lambda_1 t) - \exp(-\lambda_2)\} \quad (12)$$

The two relaxation times λ_1 and λ_2 and the numerical constant α are given by

$$\lambda_1 = (p + q)/2, \lambda_2 = (p - q)/2 \quad (13a)$$

$$\left.\begin{aligned} p &= k_{nd} + k_{nr} + k_{NN} \\ q^2 &= p^2 - 4\{(k_{nd} + k_{nr})k_{NN} + k_{nr}k_{ld}\} \end{aligned}\right\} \quad (13b)$$

$$\alpha = (k_{nd} + k_{nr} - \lambda_2)/(\lambda_1 + \lambda_2) \quad (13c)$$

Putting eq 11 and 12 into eq 10b and using the initial condition $\chi_d(t = 0) = 0$, we obtain the solution for χ_d as

$$\chi_d = [\alpha(\lambda_1 - k_{nr})/(k_{dr} - \lambda_1)][\exp(-\lambda_1 t) - \exp(-k_{dr}t)] + [(1 - \alpha)(\lambda_2 - k_{nr})/(k_{dr} - \lambda_2)][\exp(-\lambda_2 t) - \exp(-k_{dr}t)] \quad (14)$$

Finally, the expression for χ_r is obtained from eq 10c with use of eq 11 and 14, or simply from the relation $\chi_n + \chi_d + \chi_r = 1$, as

$$\chi_r = (\alpha/\lambda_1)[k_{nr} + k_{dr}(\lambda_1 - k_{nr})/(k_{dr} - \lambda_1)][1 - \exp(-\lambda_1 t)] + [(1 - \alpha)/\lambda_2][k_{nr} + k_{dr}(\lambda_2 - k_{nr})/(k_{dr} - \lambda_2)][1 - \exp(-\lambda_2 t)] + [\alpha(\lambda_1 - k_{nr})/(k_{dr} - \lambda_1) + (1 - \alpha)(\lambda_2 - k_{nr})/(k_{dr} - \lambda_2)] \times [1 - \exp(-k_{dr}t)] \quad (15)$$

Apparently the initial time derivatives of $\chi_d + \chi_r$ or $1 - \chi_n$ and of χ_d are given by $k_{nd} + k_{nr}$ and k_{nd}, respectively, as verified by the Taylor expansion of eq 10 and 13. Hence the estimates of the rate constants k_{nd} and k_{nr} from the early stage of the reaction, the procedure used in the previous section, are consistent with the present scheme.

In considering the variations of χ's with time, we have to take the end effect into consideration for the following two reasons. First, the dependences of k_{nr} and k_{nd} on the molecular weight should be considered. Second, it is seen that a much greater value of k_{nr} than that estimated from the initial slope has to be used to fit the data. Here we assume that an end behaves like one end of a denatured loop. This assumption is reasonable because the fraction of total time that a base pair is separated should be greater for base pairs near the end than for base pairs in the middle. The terminal base pairs are not really separated, however. We express the rate constant of the denaturation from one end of a loop k_{ld} as in the previous treatment and represent the greater rate expected for the denaturation from a chain end relative to that from one end of a loop by fk_{ld}. The contribution of chain ends and single chain breaks can be taken into account by allowing the initial value of N, denoted as n, to be the number of ends of both kinds (the end of a double strand counting a 0.5 and a single chain break counting as 1) divided by the number of base pairs in the molecule. The modification of the differential equations and calculations are straightforward and we obtain the expressions for χ_n and χ_d as

$$\chi_n = \beta \exp(-\mu_1 t) + (1 - \beta) \exp(-\mu_2 t) \quad (16)$$

$$N = \frac{\mu_1 - (k_{nd} + k_{nr})}{(1 + f)k_{ld}}\beta \exp(-\mu_1 t) + \frac{\mu_2 - (k_{nd} + k_{nr})}{(1 + f)k_{ld}}(1 - \beta) \exp(-\mu_2 t) \quad (17)$$

$$\chi_d = A_d[\exp(-\mu_1 t) - \exp(-k_{dr}t)] + B_d[\exp(-\mu_2 t) - \exp(-k_{dr}t)] \quad (18)$$

The numerical constants in the above equations are given by

$$\mu_1 = (u + v)/2, \mu_2 = (u - v)/2 \quad (19a)$$

$$\left.\begin{aligned} u &= k_{NN} + k_{nd} + k_{nr} \\ v^2 &= u^2 - 4\{(k_{nd} + k_{nr})k_{NN} + (1 + f)k_{ld}k_{nr}\} \end{aligned}\right\} \quad (19b)$$

$$\beta = \frac{[k_{nd} + k_{nr} + n(1 + f)k_{ld}] - \mu^2}{\mu_1 - \mu_2} \quad (19c)$$

$$\left.\begin{aligned} A_d &= \left[k_{nd} + \frac{\mu_1 - (k_{nd} + k_{nr})}{1 + f}\right]\frac{\beta}{k_{dr} - \mu_1} \\ B_d &= \left[k_{nd} + \frac{\mu_2 - (k_{nd} + k_{nr})}{1 + f}\right]\frac{1 - \beta}{k_{dr} - \mu_2} \end{aligned}\right\} \quad (19d)$$

The initial time derivatives of the three quantities χ_n, χ_d, and χ_r are given by

$$\left.\begin{aligned} (d\chi_n/dt)_{t=0} &= -[k_{nd} + k_{nr} + n(1 + f)k_{ld}] \\ (\alpha\chi_d/dt)_{t=0} &= k_{nd} + nk_{ld} \\ (d\chi_r/dt)_{t=0} &= k_{nr} + nfk_{ld} \end{aligned}\right\} \quad (20)$$

Therefore, the linear dependence of both $(d\chi_d/dt)_{t=0}$ and $(d\chi_r/dt)_{t=0}$ on the inverse of the molecular weight observed on DNase-treated poly(dA-T) is explained by the present treatment.

The direct comparison of the experimental data of χ's to the theoretical expressions, eq 16 and 17, and the estimates of the numerical values of the parameters can be made simply and accurately if we consider the conditions to be satisfied

among various parameters. That is, (1) μ_1 is larger than μ_2 because both u and v are positive; (2) β should be smaller than unity, and hence μ_1 should be greater than $(k_{nd} + k_{nr} + n(1 + f)k_{1d})$, because χ_n should be always positive; (3) similarly, μ_2 must be greater than $k_{nd} + k_{nr}$ to make N positive all the time.

Keeping these conditions in mind we first estimate μ_1, μ_2, and β to best fit the experimental data of χ_n using eq 19c and the numerical value of $(k_{nd} + k_{nr} + n(1 + f)k_{1d})$ estimated from the initial time derivations of χ_n. Second, we estimate A_d and B_d using μ_1 and μ_2 thus determined and the experimental value of k_{dr}. The comparison of the theory and experiment is made on poly(dA-T) at 34.2 and 39.8°, for illustration. The solid lines in Figure 7 are calculated using $\mu_1 = 0.101$, $\mu_2 = 0.065$, $\beta = 0.985$, $A_d = -1.02$, and $B_d = -0.0161$. Similarly the solid lines in Figure 8 are calculated using $\mu_1 = 1.96$, $\mu_2 = 0.0426$, $\beta = 0.945$, $A_d = -0.924$, and $B_d = -0.522$. Unfortunately, the estimates of the numerical values of the interesting parameters such as k_{NN} and k_{1d} from these results are not possible.

Needless to say, we cannot exclude the possibility of other reaction mechanisms. However, it can be shown that the present experimental results cannot be explained if we do not take the reaction between bases in the double-helical DNA and formaldehyde, which is possible due to the breathing of the base pairs, into account. For example, if we assume that the reaction and denaturation take place only from ends and single-stranded breaks, the decrease in χ_n is characterized by a single relaxation time. This result, of course, does not conform with the experimental results. Thus we conclude the idea of breathing of base pairs is essential in the explanation of the present kinetic results. The assumptions are: (a) the native double-helical DNA can react with formaldehyde due to the breathing of the base pairing; (b) a hydroxymethylated base pair induces spontaneous denaturation of adjacent base pairs; (c) the rate of fusion of unbonded loops is proportional to the number of loops in a molecule; (d) base pairs adjacent to loops, duplex ends, and single-stranded breaks are more reactive with formaldehyde than the base pairs in the middle of duplex DNA; (e) base pairs separate at a certain rate from ends of denatured loops and of linear duplex, and at the single-stranded breaks.

Conclusion

The new findings elucidated from the present kinetic studies of the reaction between double-helical DNA and formaldehyde at pH 9.1 are summarized as follows. (1) The hyperchromicity measurements of ultraviolet absorption at two wavelengths λ_s and λ_f make possible the separate measurements of the extents of reaction and of denaturation. (2) Reacted base pairs not only lose the hydrogen-bonding capability but also induce the denaturation of adjacent base pairs. This induction effect increases with temperature. Therefore, χ_d assumes a maximum value at some time after the initiation of the reaction. (3) Both the induction effect parameter ξ and the parameter θ, which expresses the instantaneous degree of denaturation, show a sharp increase with temperature, but it is less sharp than the increase in the optical density observed on the thermal helix–coil transition of DNA. (4) The parameters ξ and θ are smallest for T7 DNA, suggesting the long, completely matched duplex DNA is most protected against the attack of formaldehyde. (5) The kinetic equations derived on the assumptions stated previously explain the time course of the observed χ's and the molecular weight dependence of the initial rates of increase in χ_d and χ_r.

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