Kinetic Studies of the Conformation Changes of Poly(deoxyadenylate–thymidylate)†

Takayoshi Iio,‡ Thomas M. Hickey,§ Yan N. Lee,# and Eugene Hamori*

ABSTRACT: The molecular mechanisms of the formation and the disintegration of the double-helical structure of poly[d(A-T)] were investigated in a series of kinetic experiments. The conformation changes were initiated in dilute nucleic acid solutions by a pH-jump technique using a stopped-flow spectrophotometer. The absorbance (at 260 nm) *vs.* time curves characteristic to the transitions were studied under various experimental conditions (pH, nucleic acid concentration, etc.) and the results obtained (number of phases of the overall reaction, first-order rate constants, magnitudes of absorbance changes of phases, etc.) were interpreted in terms of a mechanism. According to the scheme proposed, the *formation* (from a random-coil conformation) of the double-helical structure of poly[d(A-T)] is a three-step proces involving: (1) the fast initiation and intramolecular propagation of hairpin-like branches, (2) double-helix growth starting from the convergence points of branches ("Y formation"), and (3) a minor final adjustment of the conformation by the growth of long helical branches at the expense of the very short ones. The *disintegration* of the structure (*i.e.*, the double-helix to random-coil transition) is a simple one-phase process, apparently involving the simultaneous unwinding of all helical branches. The rate of this conformation change is dependent on the average length of the helical branches present in the double-helical form. This length could be controlled by the pH at which the random-coil to double-helix conversion was carried out. Two novel experimental techniques have been used in the course of the investigations, the double pH-jump method and the computer simulation of the growth process of the double-helical branches of the nucleic acid macromolecules.

The large nucleic acid molecules of living cells often undergo intricate conformation changes when performing their biological functions. In view of the steric and topological constraints imposed on these very long polymers it is often difficult to explain these transformations in mechanistic terms. The lack of information about such conformation changes contrasts sharply with the body of accumulated knowledge about the detailed mechanism of enzymic activity and the conformational transitions of micromolecules.

A few years ago in this laboratory a kinetic investigation of the conformational transitions of the synthetic DNA analog poly[d(A-T)][1] was undertaken (Hickey and Hamori, 1971, 1972). Due to its simple primary structure (alternating sequence of A and T nucleotides; Schachman *et al.*, 1960) this poly(deoxyribonucleotide) is a very suitable model for studying the molecular mechanism of the formation and disintegration of the double-helix conformation. Although poly[d(A-T)] has not been found in nature, from certain crabs a satellite DNA has been isolated whose nucleotide sequence is almost identical (93%) with this synthetic nucleic acid (Sueoka, 1961; Laskowski, 1972). The physicochemical properties of poly[d(A-T)], including its temperature-jump-induced melting rate, have been investigated by Baldwin and his collaborators (Davies and Baldwin, 1963; Inman and Baldwin, 1962, 1964; Scheffler *et al.*, 1968, 1970; Spatz and Baldwin, 1965). In our previous publications (Hickey and Hamori, 1971, 1972) we reported a kinetic study concerned with the pH-change-induced double-helix formation of this synthetic nucleic acid. It was concluded from these investigations that under our experimental conditions the formation of the double-helical structure proceeds via a three-phase reaction (typical half-time values of the rates involved were 1 msec, 35 msec, and 3 sec, respectively). It was proposed in that work that the first phase of the reaction is associated with the development of short loops within individual macromolecules (initiation) and the subsequent spinning of these loops into hairpin like double-helical branches (propagation).

The purpose of our present investigation was to complete the kinetic investigation of poly[d(A-T)] helix–coil and coil–helix transitions by studying the nature of the second two phases of the helix *formation* process and of the entire *unwinding* process. It was hoped that the results obtained and the experimental techniques developed would lead to a better understanding of the complicated conformation changes of nucleic acid macromolecules *in vivo*.

Materials and Methods

Materials. The synthetic poly[d(A-T)] samples were purchased from Sigma Chemical Co., St. Louis, Mo., and from Miles Laboratories, Inc., Kankakee, Ill. The $s_{20,w}$ values of the

† From the Department of Biochemistry, School of Medicine, Tulane University, New Orleans, Louisiana 70112. *Received January 17, 1974.* Supported by grants from the National Institute of General Medical Sciences of the National Institutes of Health, U. S. Public Health Service (GM-18459 and 20008), and from the Petroleum Research Fund, administered by the American Chemical Society. Part of the work was completed at the Department of Chemistry, University of Delaware, Newark, Del. This paper is the third in series; parts I and II are cited in the references.

‡ Present address: Department of Physics, Faculty of Science, Nagoya University, Nagoya, Japan.

§ Present address: Department of Biochemistry, The J. Hillis Miller Health Center, University of Florida, Gainesville, Fla. 32610.

Present address: Department of Chemistry, S.U.N.Y., College of Forestry, Syracuse, N. Y. 13210.

[1] Abbreviations used are: poly[d(A-T)], alternating copolymer of deoxyriboadenylic and deoxyribothymidylic acids; t_a, aging time; t_f, average flow time: k_f, k_i, and k_s, respective rate constants of the fast, intermediate, and slow phases of the double-helix formation process; r_f, contribution (in percentage) of the fast phase to the total conformation change; pH_m, pH value of the random-coil to double-helix transition; pH_{conv}, pH value at which the double-helical sample was prepared from a random-coil sample; pH_i and pH_f, respective values of the initial and final pH before and after a pH-jump experiment; k_u, rate constant of the unwinding reaction of the double-helical nucleic acid sample.

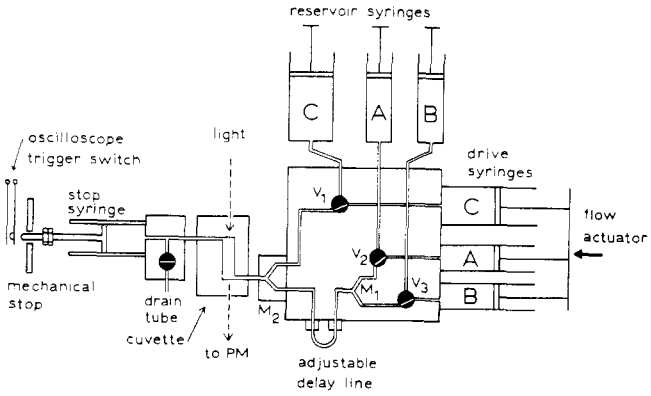

FIGURE 1: Schematic diagram of the multimixing stopped-flow apparatus used in the double-pH-jump experiments.

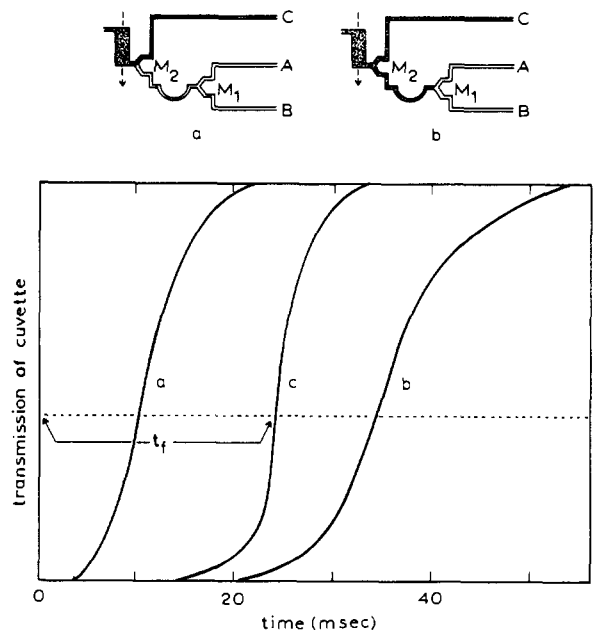

FIGURE 2: Determination of the flow time of the solution between the two mixing jets (M1 and M2 on Figure 1) of the multimixing stopped-flow apparatus. See text for details.

polymers, as reported by the manufacturers, were: sample 3, 9.23 (Miles, lot. no. 11-22-317); sample 4, 13.07 (Miles, 28-11-317); sample 5, 6.10 (Miles, 25-11-317); sample 6, 8.68 (Sigma, 90C-9010); sample 7, 11.75 (Sigma, 22C-1961-9). One sample (5) was tested in our laboratory using an analytical ultracentrifuge and was found to have an $s_{20,w}$ value (6.36 S) close to that given by the supplier. All the common chemicals used were reagent grade. The aqueous solutions were prepared with distilled water which was degassed under vacuum.

Solutions. Stock solutions of poly[d(A-T)] were prepared by dissolving a certain amount of the polymer (a lyophilized powder) in 0.0025 M NaCl. They were stored in a freezer. Poly[d(A-T)] solutions of desired polymer concentrations were made by using appropriate aliquots of the thawed stock solution and 1 M NaCl and 0.016 M Na_3PO_4. Our previous experiments have shown that the freeze–thaw treatment did not affect the properties of the solutions (Hickey, 1972).

pH Determinations. A Beckman expandometric pH meter (Model SS-2) was used to adjust the pH of the solutions, according to a procedure previously described (Senior *et al.*, 1971; Hickey and Hamori, 1972; Iio *et al.*, submitted for publication). If the pH values, measured before and after a given experiment, differed more than 0.03 unit the results were discarded.

Absorbance Measurements. These determinations were carried out either in a Cary 14 or in a Beckman DU spectrophotometer both of which were equipped with thermostated cell blocks. In some cases the Durrum-Gibson stopped-flow instrument was also used as a spectrophotometer for static absorbance measurements.

Kinetic Measurements. The rate of conformation change of poly[d(A-T)] was measured in a Durrum-Gibson stopped-flow apparatus (Durrum Instruments, Inc.) by creating a rapid pH change in the polymer solution and monitoring the ensuing hypochromicity change at 260 nm. In the *single-pH-jump* experiments the procedure used was analogous to that described in detail previously (Hickey and Hamori, 1972). The *double-pH-jump* experiments were carried out in the following manner. The valve block assembly of the standard Durrum-Gibson stopped-flow chamber unit was replaced by a multimixer valve block assembly (Model D-132 without collection block and electronic control unit) resulting in the apparatus shown schematically in Figure 1. With this modified version of the stopped-flow instrument it was possible to initiate *two* fast pH changes in rapid succession in poly[d(A-T)] solutions. The first pH jump was brought about by the mixing of the polymer solution (A in Figure 1) with the first buffer solution (B) at the first mixing jet (M1), and the second, by the mixing of the resulting polymer solution (*i.e.*, A + B) with the second buffer (C) at the second mixing jet (M2). The progress of the conformation change of the polymer triggered by the *second* pH jump was monitored spectrophotometrically at 260 nm in the cuvet attached directly after the second mixing jet. The time period between the first and second pH jumps (aging time, t_a, to be discussed in detail below) could be controlled within certain limits depending on the operational mode of the experiment. In the *single-activation* mode the drive syringes were operated only once and the aging time was controlled by adjusting the length of the delay line between the two mixing jets. In this operational mode the shortest obtainable aging time was 19.5 msec (corresponding to the shortest possible delay-line length) and the longest 48.5 msec. (The limiting factor for long aging times was the maximum adjustable travel distance of the stop-syringe plunger; the amount of liquid which is forced through the delay line could not exceed the travel volume of this plunger—0.6 ml in the present construction of the instrument.) In the *double-activation* mode the drive syringes were operated twice in succession. During the first activation the delay line was filled with a polymer solution whose pH had been changed as a result of the first mixing at M1. After the elapse of the desired aging time the drive syringes were operated again the second time. In this latter operation poly[d(A-T)] which was exposed for the duration of the aging time to a certain pH environment would be mixed with the second buffer solution at the second mixing jet and the ensuing conformation change would be observed immediately in the adjoining spectrophotometric cuvet. The lower limit of the aging time in this particular operational mode was determined mainly by the dexterity of the operator in rapidly resetting the stop-syringe plunger after the first activation step (this adjustment required about 1.5 sec for us) but there was no apparent upper limit on the duration of the aging time.

The dead time[2] of the standard valve block assembly which was utilized in the single-pH-jump experiments was determined to be 3 msec at 20° and 6 msec at 10° using $Fe(NO_3)_3$

[2] The dead time of a stopped-flow instrument is the period elapsed between the extrapolated starting time of the reaction and the stabilization of the transmission signal of the observation cuvet at a value free of mixing (or other) artifacts.

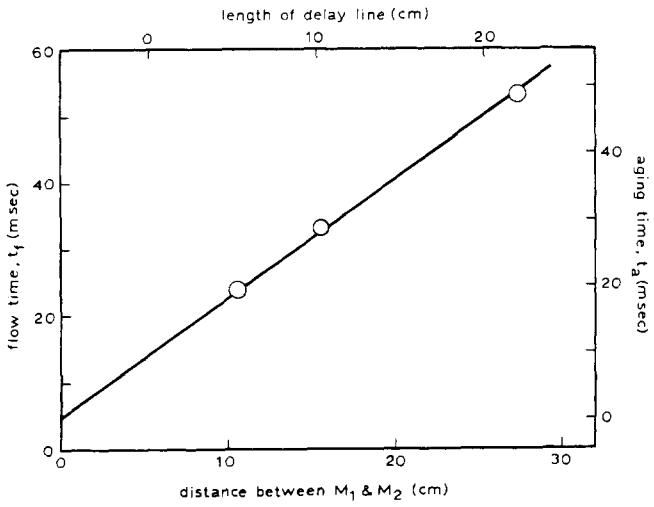

FIGURE 3: Calibration curve relating the delay line length (and M1-M2 distance) of the multimixing unit to the flow time (and aging time) of the solution. See text for further details.

and KCNS solutions in a procedure previously described (Hickey and Hamori, 1971; Hickey, 1972). The dead time of the multimixer valve block assembly used in the double-pH-jump experiments was found to be 14 msec at 10°. In the latter dead time determinations syringes A and B (Figure 1) contained $Fe(NO_3)_3$ solutions and syringe C the KCNS solution; thus the dead-time value observed under these conditions is determined by the efficiency of the operation of the second mixing jet (M2) and the adjoining spectrophotometric cuvet.

In most experiments involving the multimixing unit it was important to know the exact time required for the reactant solution to flow from the first mixing jet through the delay line to the second mixing jet. The relationship between this time and the adjustable length of the delay line was established as follows. The cuvet of the stopped-flow instrument and the flow lines leading from the cuvet to syringe C were filled with dilute iodine solution ($A_{450} \simeq 0.5$). Syringes A and B and the lines leading from these syringes to mixing jet M2 were filled with water (Figure 2, drawing a). The system so prepared was activated and the transmission changes of the cuvet were recorded on the oscilloscope which was triggered at the very beginning of the flow. Curve a of Figure 2 shows a typical result; the transmission change seen reflects the dilution of the iodine solution at mixing jet M2. The sigmoidal character of the curve is due to transient effects (flushing of lines and cuvet, etc.) which occur between the mixing jet M2 and the outlet of the cuvet. (These effects are related to the dead time of the instrument.) In another experiment the lines of the valve block were filled with iodine solution and water as before, except that in this case, the line between mixing jets M1 and M2 contained iodine solution and not water as in the previous experiment (Figure 2, drawing b). Following the activation of the instrument, the transmission of the cuvet was recorded again from the onset of the flow (Figure 2, curve b). The result obtained was a dilution curve of the iodine solution for the case when water starts from point M1. In order to correct for the transient effects between point M2 and the cuvet, which are also included in the latter oscilloscope trace, the abscissa values of curve a were subtracted from those of curve b resulting in the transmission curve c. Apparently owing to a slight mixing of the iodine solution and the incoming water in the line section M1-M2, the transmission change indicated in curve c was still not an abrupt step function but also a sigmoidal curve. Assuming that this curve reflected the (directly unobservable) transmission changes at point M2, curve c was used for the determination of the flow time of the solution from point M1 to point M2. The average flow time (t_f) of the water molecules from mixing jets M1 to M2 could be obtained from the results by determining the time required for the absorbance of the cuvet (calculated from curve c) to reach a halfway mark between the initial and final values (broken line, Figure 2). By establishing t_f data in this manner for three different delay-line lengths a calibration curve (Figure 3) could be constructed for t_f values in terms of M1-M2 distances. (Note that in the multimixing unit M1-M2 distance equals 5.3 cm + delay-line length.) Since in all these experiments the recording of the transmission changes started before the flow could establish a steady velocity, the data shown in Figure 3 do not extrapolate to zero t_f value at zero M1-M2 distance. In order to compensate for this "starting effect," which is absent in single-activation pH-jump experiments, the desired aging times (t_a) were calculated by subtracting the ordinate intercept value from the t_f data. The y axis of the aging time scale on the right side of Figure 3 was positioned taking this correction into account.

TABLE 1: Values of the Rate Constants k_i and k_s at Different Polymer Concentrations.[a]

Poly[d(A-T)] Concn (μg/ml)	k_i (sec^{-1})	k_s (sec^{-1})
5.0	4.6 ± 0.3	0.33 ± 0.01
15.0	4.7 ± 0.9	0.32 ± 0.03
30.0	4.6 ± 0.4	0.31 ± 0.05

[a] The pH values of the solutions before and after mixing were 12.30 and 11.86, respectively. The polymer used was sample 7.

For all experiments the temperature of the solution in the stopped-flow apparatus was maintained at 10.00 ± 0.05° by a refrigerated circulator bath (Forma Scientific, Inc., Model 2096). The light source for the monochromator of the stopped-flow instrument was a Beckman deuterium lamp (No. 96280) powered by a Beckman hydrogen lamp power supply (Model B). The transmission changes of the solutions were displayed and recorded on a Tektonix dual-beam storage oscilloscope (Model 5103N/D13) equipped with a high gain differential amplifier (5A20N) and two time-base generators (5B10N). The modules of the oscilloscope were connected in such a manner that the two electron beams of the cathode ray tube could be controlled independently by the two time-base generators. For the evaluation of the rate constant data the oscilloscope traces were photographed (Tektronix camera C-5) and replotted on semilogarithmic graphs.

Results

Studies of the Rates of Double-Helix Formation. CONCENTRATION DEPENDENCE. We reported in a previous paper (Hickey and Hamori, 1972) that at low polymer concentrations the variation of the concentration of poly[d(A-T)] has no effect on the rate constant (k_f) of the fast phase of the double-helix formation process. In our present investigation we carried out a similar study on the intermediate and slow phases. The results summarized in Table I indicate that the rate constants k_i and k_s are also concentration independent.

pH DEPENDENCE. Figure 4 shows the variation of the rate constants k_i and k_s with the initial and final pH values of the solutions. It can be seen that while the values of the initial pH have no apparent effect on the rates, decreasing final pH values

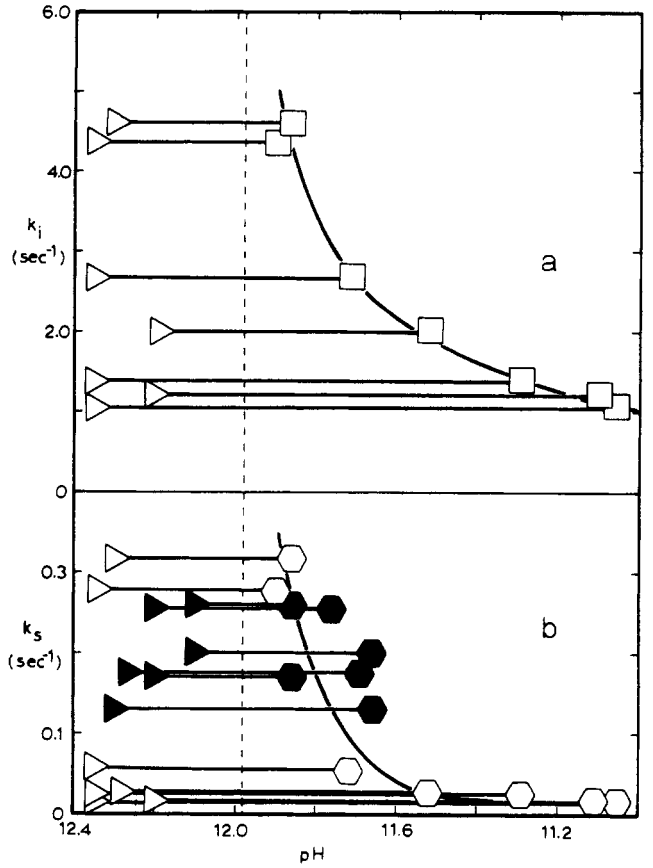

FIGURE 4: The effect of pH on the intermediate (a) and slow (b) phases of the double-helix formation process. The reactions initiated by rapid pH changes in the stopped-flow apparatus are represented by horizontal lines in this graph. The left ends of these lines (△) show the initial pH values of the solutions before mixing and the right ends (□ or ○) the final pH after mixing. The ordinate values of the lines represent the rate constants observed. The vertical dotted line is the value of pH_m under the experimental conditions used (10°, 25 mM Na^+, 4 mM PO_4^{3-}, 12.5 μg/ml of polymer). The polymer samples used were: 7 (open symbols) and 4 (filled symbols).

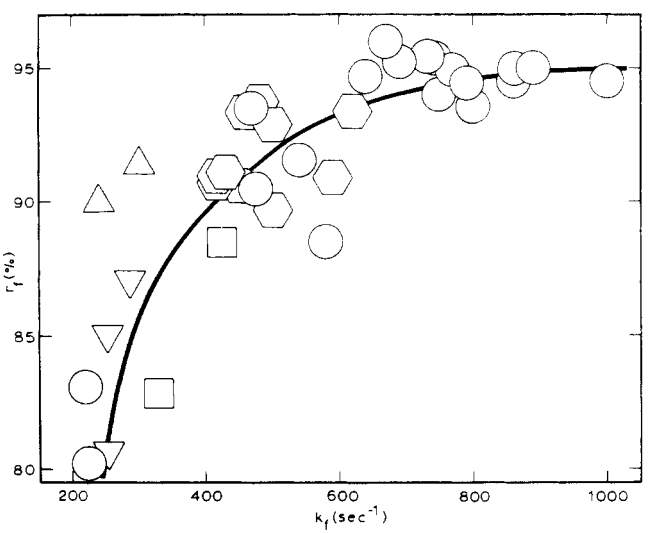

FIGURE 5: Correlation between the rate of the fast phase of helix formation (k_f) and the percentage of this phase in the total conformation change (r_f%). The regular data are designated by circles. The symbols ⬡, □, and ▽ respectively represent data obtained in the presence of glycerol, Acridine Orange, and carboxymethylcellulose; △ indicates formaldehyde treatment. Details of the experimental conditions have been described either in the text or have been published elsewhere (Hickey and Hamori, 1972; Hickey, 1972).

decrease significantly both k_i and k_s. These results are in marked contrast to the previously observed increase of k_f with decreasing final pH values (Hickey and Hamori, 1972).

PERCENTAGE OF THE FAST PHASE. Figure 5 indicates the dependence of the percentage of the fast rate in the total conformation change r_f (%), on the magnitude of k_f. In spite of the scatter of data, a correlation can be detected between r_f and k_f values in the k_f region below 600 sec^{-1}.

SPECTROSCOPIC STUDIES. In order to test whether the three phases of the double-helix formation process represent an identical chemical reaction proceeding at different rates, spectroscopic studies were undertaken. Using various settings of the monochromator of the stopped-flow instrument the progress of the reaction was monitored at eight different wavelengths from 235 to 265 nm. From the recorded data several curves could be constructed representing the absorption spectrum of the system at various time intervals from the start to the completion of the conformation change. These curves (not shown) indicated that the absorption changes strictly in proportion over the entire wavelength range during the fast and intermediate phase of the conformation change. For the slow phase, however, there was some indication that the slow rate (k_s) might be slightly faster when observed in the neighborhood of 235 nm than when monitored around 260 nm.

SIMULATION OF DOUBLE HELIX FORMATION IN A DIGITAL COMPUTER. In order to test whether the random-nucleation and branch-twisting mechanism proposed for the fast phase of poly[d(A-T)] double helix formation (Hickey and Hamori, 1972) could result in the first-order process observed, we simulated this conformation change using a digital computer (Burroughs 6700) according to the following scheme: The nucleotide units in P polymer molecules were defined by $N = PD$ integers (D is the number of nucleotide units per molecule), and random nucleation and the subsequent growth of double-helical branches was generated in this system by the computer. A nucleus was defined to be an incipient double-helical branch with four unpaired nucleotides in the loop and four nucleotides in the stem forming two base pairs. In each cycle of the program the rate of nucleation (number of nuclei generated per cycle) was kN_r, where k is an adjustable parameter and N_r is the number of nucleotide units still in random-coil form (*i.e.*, those which are neither in the loops nor in the base-paired regions of the polymers). The computer selected the nucleation sites randomly and unsuitable sites (*e.g.*, those which were located next to chain ends or to other nuclei) were bypassed. In each successive cycle, in addition to generating new nuclei, the computer added a new base pair to each of the existing helical branches if free neighboring nucleotides were available. The cycling stopped when all helical branches ceased growing and the rate of nucleation dropped below a set minimum level. Using values of $P = 3$, $D = 1000$, and $k = 0.01$, we found that the rate of simulated double-helix formation obeys the first-order rate law well (Figure 6). The computer printout of this particular experiment depicting the stepwise growth of helical branches in one polymer molecule is shown in Figure 7. In another simulation experiment the values of P and D were as above, but k was set to 0.001; in this case the polymer conformations generated by the computer displayed fewer and longer branches (Hickey, 1972) but the rate did not follow a first-order relationship as well as in the previous case. (See paragraph at the end of the paper concerning supplementary material.)

Investigation of the Kinetics of the Unwinding Process. GENERAL CHARACTERISTICS OF THE REACTION. If the pH of a poly[d(A-T)] solution is rapidly raised from a value below pH_m to a value above pH_m, a double-helix to random-coil con-

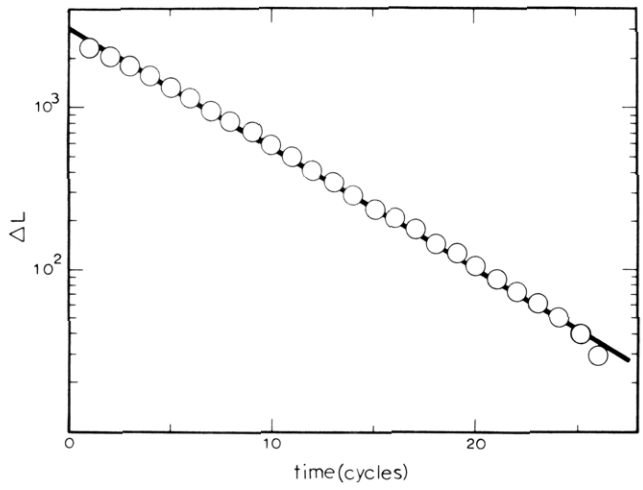

FIGURE 6: First-order plot of a computer-simulated double-helix formation. The ordinate represents the number of nucleotide units which are not parts of double-helical branches, and the abscissa the number of computer cycles (a parameter analogous to time). See text for further details.

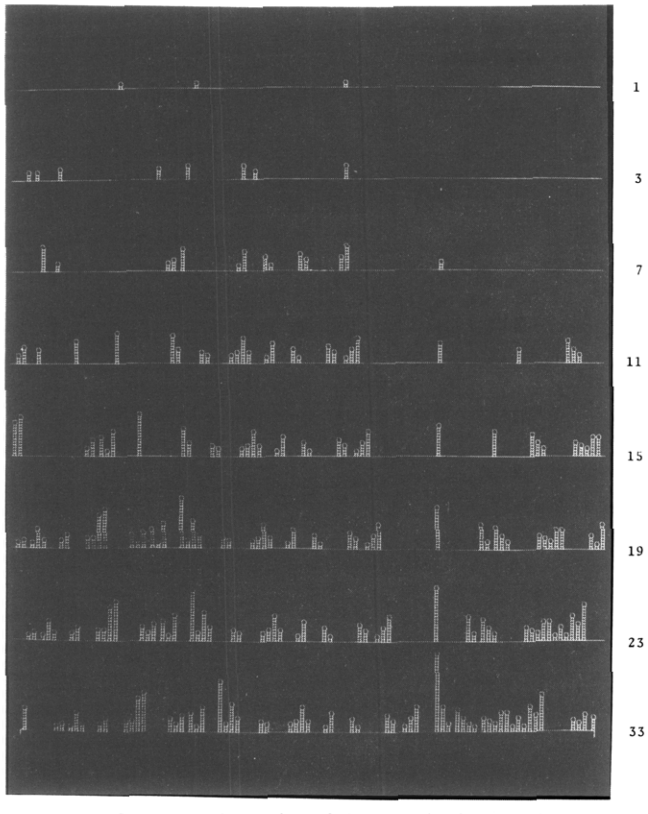

FIGURE 7: Computer simulation of the growth of helical branches on a single poly[d(A-T)] chain. The sequence of horizontal figures from top to bottom indicates schematically the development of the highly branched conformation of the macromolecule. The numbers on the right correspond to computer cycles. With the exception of the last conformation (bottom), only a part of the entire polymer chain is shown. See text for further details.

formation change will be initiated in the system which is observable by monitoring the transmission of the solution at 260 nm. A typical record of such experiment carried out in the stopped-flow apparatus is shown in Figure 8. The experimental conditions are given in the legend of this figure. As can be seen from the semilogarithmic plot of the reaction (Figure 9) the unwinding process is a single first-order reaction which extrapolates back to the initial absorbance of the system at zero time. The same kind of kinetic behavior was observed in all experiments involving the pH-change induced unwinding of poly[d(A-T)] helices.

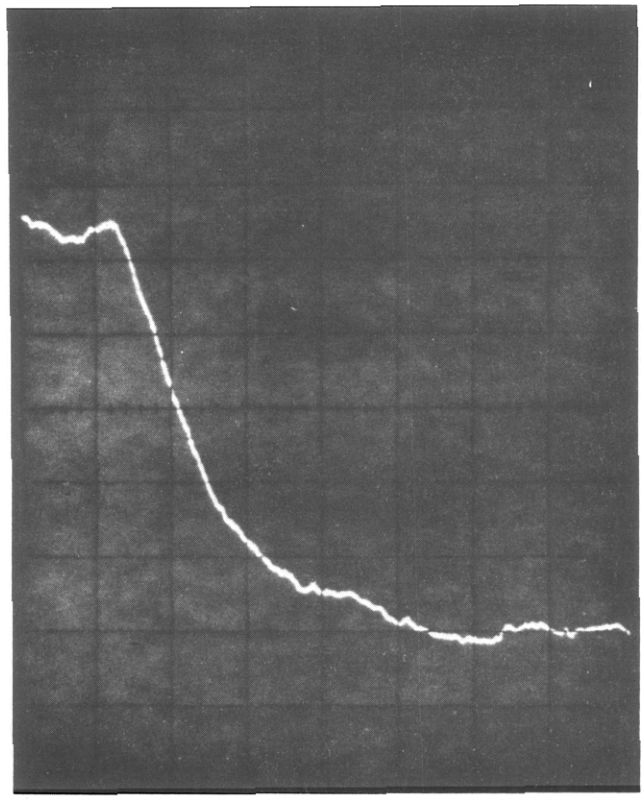

FIGURE 8: Oscilloscope record of the rapid unwinding of double-helical poly[d(A-T)]. ΔpH: 11.53 → 12.16; 25 mM Na^+: 4 mM PO_4^{3-}, 10°. The polymer sample (7) was used without any treatment; concentration 12.5 μg/ml. The horizontal scale is time (2 msec/div) and the vertical one is transmission at 260 nm.

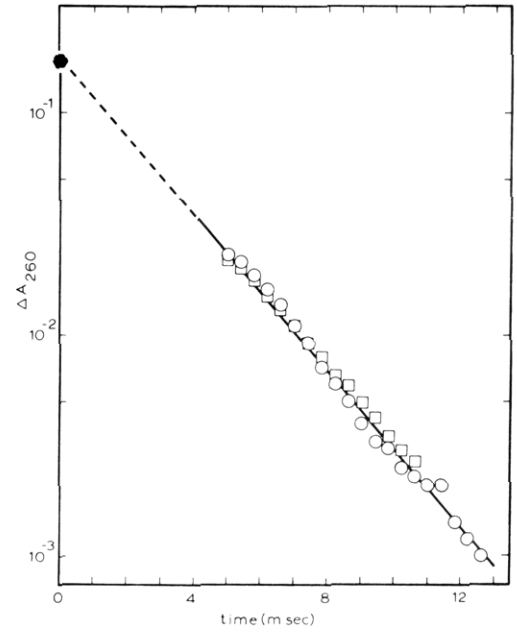

FIGURE 9: First-order plot of the process shown in Figure 8 (○) and that of a separate experiment carried out under identical conditions (□). The initial ΔA_{260} value of the systems (●) was measured independently. $k_u = 407\ sec^{-1}$.

pH EFFECTS. In order to discover whether the history of preparation of double-helical poly[d(A-T)] would affect the rate of unwinding, various samples were prepared by lowering the pH of random-coil poly[d(A-T)] solutions to different

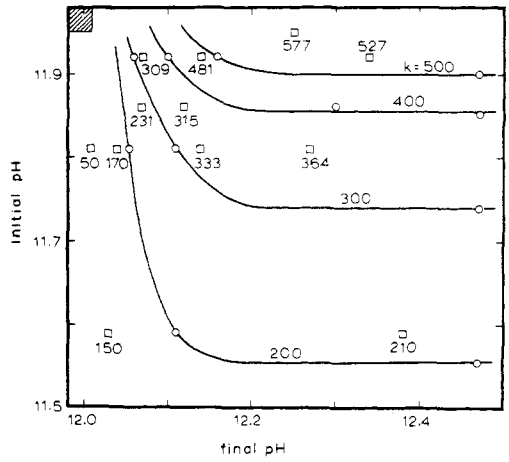

FIGURE 10: The effect of initial and final pH values on the rate constant of the double helix unwinding process. The numbers beside the symbols and lines indicate the values of rate constants (in sec^{-1} units) corresponding to certain pH_i/pH_f values. The squares represent experimental measurements and the circles and lines show interpolated results. The shaded square in the upper left-hand corner represents the transition pH region of the system. The polymer sample used was 6; the other experimental conditions were same as those of Figure 8.

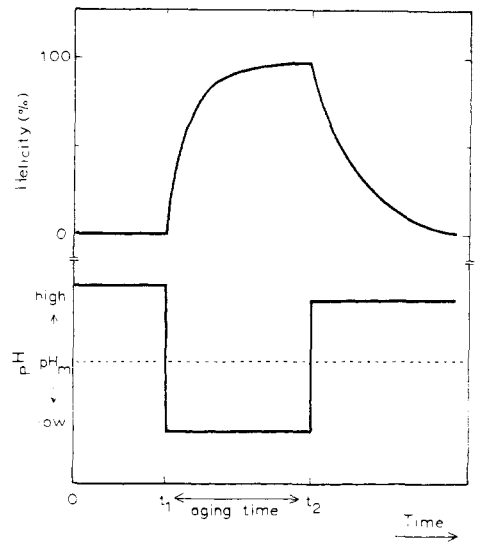

FIGURE 11: Schematic (and idealized) representation of the pH changes of the solution and the conformational transitions of nucleic acid macromolecules during a typical double-pH-jump experiment.

values below the transition pH. For better reproducibility of the results these pH changes were carried out in the stopped-flow instrument. Following this operation, the solutions were collected from the apparatus at the drain tube, their pH values were adjusted (by titration) to a common value (11.53), and they were placed back to the stopped-flow machine for the unwinding rate studies. In one of the series of experiments the adjustment of pH values from pH_{conv} to pH_i was omitted from the operations. Table II lists the experimental conditions and the results obtained in these pH-effect studies. It can be seen in the table that the unwinding rate depends significantly on the pH value at which the originally random-coiled poly[d(A-T)] was converted to double-helical form. For this reason the pH-dependence study of the unwinding rate constant (k_u) was carried out on double-helical poly[d(A-T)] samples of identical pH history. (They wers taken from the same stock solution and they were never exposed to high-pH environment prior to the unwinding experiments.) The results summarized by Figure 10 indicate the effect of the initial and final pH values of the pH jump on the rate constant of the unwinding reaction.

TABLE II: Effect of pH Value of the Random-Coil to Double-Helix Conversion (pH_{conv}) on the Unwinding Rate (k_u) of Double-Helical Poly[d(A-T)] Samples.

Series	pH_{conv}[a]	pH_i[b]	pH_f[c]	k_u (sec^{-1})
A	11.78	11.78	12.18	202
	11.54	11.54	12.14	99
B	11.89	11.53	12.16	433
	11.67	11.53	12.17	385
	11.31	11.53	12.17	318
	11.12	11.53	12.16	273

[a] The conversion from random-coil to double-helical conformation was carried out, either in the multimixing stopped-flow apparatus at mixing jet M1 (series A) or in the regular stopped-flow instrument (series B), by lowering the pH of the polymer solutions from a value above pH_m to the value designated in this column. The polymer sample used was 7. [b] The pH values of the solutions were either adjusted from pH_{conv} to pH_i by titration or were left unchanged. [c] The pH jump ($pH_i \rightarrow pH_f$) initiating the unwinding reaction was carried out in the regular stopped-flow apparatus using the conditions described in Figure 8.

MULTIMIXING EXPERIMENTS. In order to learn about the nature of the various phases of the helix formation process a series of experiments was carried out in which the unwinding of the nascent helices was measured. Using the stopped-flow apparatus equipped with a multimixing valve-block assembly, double pH jumps were executed in the solutions (see Experimental Section). In these experiments the first pH jump triggered a double-helix formation process (from random-coil polymers), and the second pH jump, initiated after a predetermined aging time (t_a), caused the unwinding of the freshly formed double-helical structures. Figure 11 is a schematic representation of the pH changes of the solution and the conformation changes of the polymer during such a double-pH-jump experiment. This technique made it possible to measure the unwinding rate (k_u) of double-helical structures at various times after their formaton was initiated. The results of these experiments are summarized in Figure 12. It can be seen that poly[d(A-T)] helices maturing through the intermediate (*ca.* 0.02–0.4 sec) and slow (*ca.* 0.4–100 sec) phases of the helix formation process unwind with progressively slower rates.

Discussion

It appears that the conformation change of poly[d(A-T)] molecules triggered by a rapid decrease in the pH of the solution involves an intramolecular progagation of a single chemical reaction. This conclusion can be drawn from the observed concentration independence of the three phases of the reaction, and from the fact that these phases are accompanied by an identical spectroscopic change. From other available evidence (Schachman *et al.*, 1960; Inman and Baldwin, 1962, 1964; Davies and Baldwin, 1963; Davidson *et al.*, 1965; Hickey and Hamori, 1972) it can be presumed that this chemical reaction is the formation of a double helix similar in structure to that of native DNA in the B form. The first phase of the conformation change has been explained in terms of the development of small loops on the macromolecule and the subsequent rapid twisting of these nuclei into double-helical hairpin-like branches (Hickey and Hamori, 1972). Based on the kinetic experiments reported in the present study, further details of the

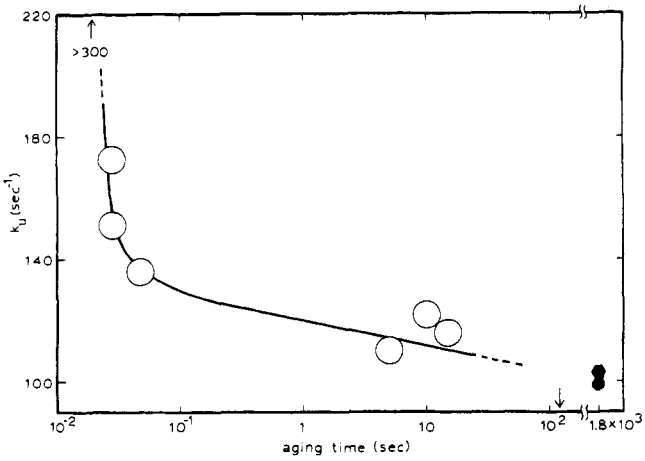

FIGURE 12: Change of the unwinding rate of double-helical poly[d(A-T)] with aging time, *i.e.*, the time elasped between the initiation of the double-helix formation and the initiation of the unwinding process. The three data points (○) on the left were obtained by the single-activation, and those on the right by the double-activation method of the multimixing technique (see Experimental Section for details). The initial, intermediate and final pH values of the solutions were 12.31, 11.53, and 12.14, respectively. The polymer sample used was 7. The data represented by solid symbols were obtained in the following manner. (●) The first pH jump (12.29 → 11.53) was carried out in the multimixing unit at mixing point M1 (see Figure 1) and the sample was removed through the opened delayline. (◐) The same pH jump was carried out in the standard stopped-flow unit and the sample was collected through the drain tube. In both of these experiments, following the above operation, the solutions were replaced into the standard stopped-flow instrument for the second pH jump (11.53 → 12.14).

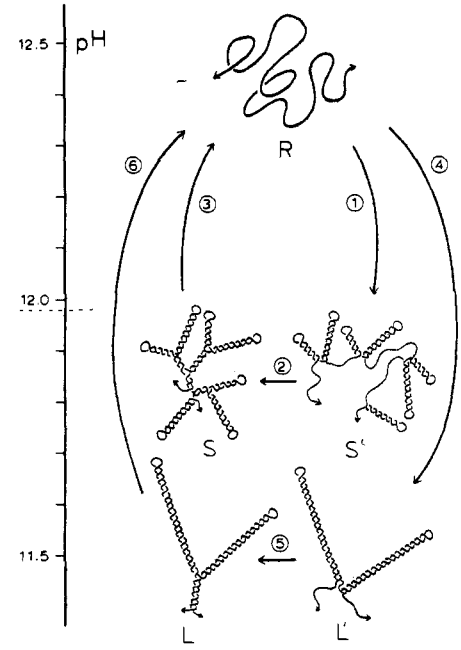

FIGURE 13: Schematic representation of the postulated conformation changes of a poly[d(A-T)] molecule induced by sudden pH changes. The vertical position of the conformations corresponds approximately to the pH of the solution. The broken line represents the transition pH of the system. See text for more details.

mechanism can be clarified. Figure 13 represents our current interpretation of the overall mechanism of this complex conformational change. In the following we shall discuss this scheme in detail.

When the pH of the solution of a randomly-coiled poly[d(A-T)] molecule Figure 13, R) is lowered below the pH_m value of the system, the charged thymine bases of the polymer will become rapidly protonated and the number of negative charges on the molecule will suddenly decrease. The extent of this charge reduction will be approximately proportional to the size of the pH jump imposed upon the solution (Hickey, 1972). The observed increase in the rate of the first phase of the double-helix formation with the size of the pH jump suggests that the rate of initiation and/or propagation of the reaction is controlled not by a hydrodynamic drag on the nucleic acid chain but by the presence of residual charges on the polymer after the pH jump. For reasons discussed below, we believe that it is the rate of *propagation* which is being controlled. Apparently, the spinning double-helical branches are arrested momentarily when they encounter a charged thymine residue which can not be incorporated into a double helix. Owing to a statistical fluctuation, however, these charges translocate from time to time, and consequently, the arrested branches can resume their growth. According to this view a small pH jump (*i.e.*, one which leaves many residual charges on the polymer) will trigger a relatively slow reaction leading to a highly branched product (arrow 1, Figure 13), but a large pH jump (fewer residual charges) will cause a relatively fast reaction leading to a less branched and more extensively double-helical product (arrow 4). It appears that the final cessation of this process is caused by the convergence of the growing double-helical branches within the macromolecules (Hickey and Hamori, 1972). After this process, designated as fast phase, a slower process, identified as intermediate phase, is observed (Figure 13, arrows 2 and 5). It is evident from the results shown in Figure 4 (curve a) that at final pH values close to pH_m the intermediate reaction proceeds faster than at low pH. In other words, the reaction indicated by arrow 2 of Figure 13 is faster than that of arrow 5. If it is assumed that this intermediate process is a further propagation of the double-helical structure by a mechanism involving the simultaneous spinning of two converged hairpin branches ("Y" formation), the observed pH dependence can be explained as follows. In one case (arrow 2), the propagation process requires the rotation of *short* pairs of hairpins, but in the other (arrow 5), it entails the rotation of *long* pairs of hairpins whose hydrodynamic resistance is higher than that of the shorter hairpins.

In addition to the intermediate reaction there is a minor (2–3%) slow phase present in the total observed conformation change. It could be speculated that this slow phase is associated with some final rearrangement of the branches on the polymer, involving perhaps the unwinding of very short hairpins and the growth of longer ones. This type of rearrangement would be expected to yield a more efficiently duplexed structure. In the conformation changes indiated by arrows 2 and 5 of Figure 13 this slow minor phase is not represented explicitly.

It can be seen in Figure 5 that under conditions favoring high k_f values (*e.g.*, large ΔpH, absence of additives, etc.) the extent of the fast phase (r_f) is larger than that under conditions resulting in a relatively slow reaction. This must mean, of course, that following the first phase of the conformation change, less extensive random-coil regions will be present on the polymer after a faster reaction (arrow 4, Figure 13) than after a slower one (arrow 1). This finding is in good agreement with our model since the unencumbered fast propagation of helical branches would be expected to lead to a product of higher helix content than that of the frequently arrested slower process (*cf.* L′ and S′ of Figure 13). Note that if k_f were controlled by the effect of residual charges on the rate of *nucleation*, the slow version of the first phase (arrow 1) would be expected to yield state L′ and the fast version (arrow 4) state S′. In this case, however, the experimentally observed effects of pH on k_i (arrows 2 and 5), and k_f on r_f (Figure 5), would not logically follow from the mechanistic model.

It will be recalled that all three phases of the double-helix formation reaction appear to follow first-order kinetic relationships. For a tentative interpretation of this behavior a mechanism might be considered involving sudden all-or-none type conformation changes of isolated poly[d(A-T)] molecules. According to this model, the altered pH milieu would make the random-coil conformation of the polymers unstable and they would spontaneously (and essentially completely) convert to the stable (double-helical) conformation in a true unimolecular manner. We find this mechanism unattractive for the following reasons. The observed three distinct phases of the reaction could not be readily explained by this mechanism (a little reflection will reveal that if the fast phase were due to such mechanism it would become a slower and slower process *gradually* and not a stepwise manner); the observed pH dependence of k_i and k_s data could not be patently interpreted, and finally, the highly branched conformation of the poly[d(A-T)] molecules observed under the electron microscope (Davidson *et al.*, 1965) would not follow directly from this model. Therefore, in contrast to this mechanism, we believe, that the double-helix formation of poly[d(A-T)] involves the *de facto* simultaneous conformation change of all macromolecules according to the scheme put forth above (Figure 13). The drawing derived from our computer-simulation studies (Figure 7) demonstrates the beginning steps of this mechanism in an illustrative manner. (Note that the "Y-formation" process has not been incorporated into the simulation.) According to this mechanism the reaction is initially fast (discounting a brief incubation period) because many branches nucleate and grow. Later, due to the diminishing of available nucleation sites and the stopping of most growing helical branches the rate decelerates. The first-order character of the process appears to be the consequence of a proportionality between the reaction rate and the number of growing branches. The explicit mathematical treatment of this reaction mechanism is rather difficult but the computer simulation experiments clearly indicate the possibility of a first-order type relationship (Figure 6). (It must be noted that due to the experimental errors and the difficulties associated with precise separation of the three phases of the reaction, our results can rule out only a gross deviation of the respective phases from first-order behavior. It appears likely that these rates are not actual first-order processes but only closely resemble first-order processes.)

We shall proceed now to discuss the results obtained on the rate of *unwinding* of double helical poly[d(A-T)]. In our experiments this conformation change was initiated by suddenly raising the pH of a solution from a value below pH_m to that above pH_m. As indicated by the typical results shown in Figures 8 and 9, the observed absorbancy changes were simple exponential processes. The obvious lack of multiphase character of the curves suggests that, after the pH jump, the unwinding of helical branches proceeds in all regions of the molecule without any preliminary conformation change. The first-order nature of the transition could be due to an approximate proportionality between the rate of absorbance change and the number of helical branches participating in the reaction.

According to our results (Figure 10) the first-order rate constants of the unwinding rates are between 50 and 500 sec^{-1}. These values fall within the broad range of rate constant values ($10-10^4$ sec^{-1}) found by Spatz and Baldwin (1965) for the temperature-jump-induced melting rates of double-helical poly[d(A-T)].

The effect of the final pH value of the pH jump (pH_f) on the unwinding rate can be predicted by considering the influence of the negative charges present on an unwinding poly[d(A-T)] molecule. It would appear that the electrostatic repulsion among these charges would accelerate the separation of the strands of the helical branches, and accordingly, pH jumps to higher pH values (more charges) would result in faster rates than those to lower pH values (fewer charges). The inspection of the data of Figure 10 along horizontal lines (constant pH_i values) will reveal that the observed rates follow this predicted behavior.

In addition to the pH of the solution during the unwinding reaction, the specific conformation of the double-helical poly[d(A-T)] molecules at the onset of the reaction can also be expected to influence the unwinding rate. This effect is shown in the simplest form by the results of series A in Table II, which indicate that under practically identical pH_f conditions the S-type conformation formed at pH 11.78 unwinds faster than the L conformation formed at pH 11.54. This is in a good agreement with the simple unwinding mechanism discussed above, since the conformation change of the S form proceeding *via* the simutaneous unwinding of the numerous branches must be faster than that of the L form containing fewer branches (*cf.* arrows 3 and 6 in Figure 13). The other results of Table II (series B) show a further effect which complicates this simple view. It is indicated in that table that an S-type polymer formed at pH value slightly under pH_m (11.89) does not unwind with a slow rate characteristic to the L form when the pH of the solution is changed (prior to the pH jump) to a value favoring the L form (pH_i = 11.53). Apparently, the conversion of the S form to the slightly more thermodynamically stable L form is infinitely slow. (The activation energy for this process must be very high since it has to involve the unwinding of helical branches of substantial length.) It can be seen from the rest of the results of series B in Table II that at constant pH_i and pH_f values the unwinding rates decrease steadily when the pH_{conv} of the double-helical structures decrease. Apparently, under these experimental conditions, the conformations of the macromolecules, which are set at their formations by their respective pH_{conv} values, determine the unwinding rates.

The comparison of the absolute values of the results of series A and B in Table II (and also of Figure 10, discussed below) will reveal some unexpected differences. We believe that these inconsistencies are due to the different conditions which were used in these experiments. The unwinding rates being strongly dependent on the specific conformations of the double-helical poly[d(A-T)] molecules are apparently very sensitive to even minor changes in the experimental techniques involved.

The results shown in Figure 10 were obtained on polymer samples which had never been exposed to high pH environment, excepting, of course, the (upward) adjustment of the pH of the solution to the value of pH_i immediately prior to the stopped-flow experiments. It can be seen in this figure that the observed k_u values increase with pH_i along any vertical (pH_f = constant) line. Apparently, when the poly[d(A-T)] macromolecules in the L state[3] are exposed to a pH environment which is only slightly below pH_m they convert to the S form and exhibit the fast unwinding rate which is associated with this form. The driving force for this transition would be the increased number of end loops in the S state which, being in the random-coil form, can easily accommodate the negative charges which develop on the polymers at these pH values. (Our model-building studies with CPK atomic models have indicated that the freely

[3] The state of branching of the commercial poly[d(A-T)] samples we used is not known with certainty, but the electron micrographs of similar preparations (Davidson *et al.*, 1965) suggest that it is closer to our formulated L state than to the very highly branched S state.

rotating bases at the ends of hairpins are able to assume distant positions, and in this manner, the electrostatic repulsion among the negative charges can be minimized.) Spatz and Baldwin (1965) reported that the temperature-jump-induced melting rates of double-helical poly[d(A-T)] samples increased when the starting temperature of the solutions approached the melting temperature of the polymers. Our observed increase of k_u with pH_i (Figure 10) is analogous to these findings.

Thus, our studies on the unwinding rates of double-helical poly[d(A-T)] can be summarized by stating that for polymer samples of identical "starting" conformation k_u increased with pH_f (effect of negative charges), but if the conditions of the pH jump are the same the starting conformation (determined by pH_{conv} and/or pH_i) will control the rate (S form will unwind faster than L form; see arrows 3 and 6 in Figure 13).

It is to be noted that the observed dependence of unwinding rate on the specific conformation of the double-helical poly[d(A-T)] sample is an indication against the possibility that the fast hypochromicity change observed during the presumed disappearance of the helical structure is not an unwinding, but rather a preliminary disintegration process (involving, for instance, the breakage of hydrogen bonds and the "flipping out" of bases from the center toward the outside of the helix) which is subsequently followed by (a spectroscopically unobservable) slower unwinding of the still entwined strands.

The results of the double-pH-jump experiments (Figure 12) indicate that the helical structures formed before the onset of the intermediate phase of the reaction unwind with very high rates whose measurement is beyond our instrumental capability. The unwinding rate decreases during the intermediate phase and reaches a plateau value which is not changed much during the slow phase of the reaction. Thus, the preliminary findings of this difficult experimental technique suggest that the short helical branches not yet tied together by the Y-formation mechanism can unwind very rapidly (in Figure 13 the intermediate states between forms R and S′ or R and L′ would correspond to these polymer conformations) but the overall rate of the unwinding reaction slows down when the conformations go through our postulated Y-formation process. The conformation change which occurs during the slow (third) phase of the double-helix formation process has a relatively minor effect on the unwinding rate. This is in agreement with our conclusion that this phase is not a major conformation change (accompanied by a small hypochromicity, conceivably) but rather some limited optimization of the existing conformation.

Several studies have been published in recent years, most notably from the laboratories of Eigen and Crothers, concerning the kinetics of helix–coil transitions of synthetic polynucleotides. Many of these investigations were based on temperature-jump relaxation experiments using short oligomeric materials and lead to the elucidation of the mechanism and rate constants involved in the *elementary steps* (*e.g.*, base pairing or dissociation) of the helix–coil transitions (Pörschke and Eigen 1971; Craig *et al.*, 1971). Our studies reported here and in our previous paper represent an approach different from the above investigators with the consequence of relatively little overlap between the research findings. In our studies, for instance, owing to the unknown number of growing helical segments we were unable to derive rate constant values for the propagation of the helical structure per active helical segment. On the other hand, our investigations have been able to shed more light on the intricate mechanism of a *drastic* conformation change of a high molecular weight nucleic acid than typical relaxation-kinetic experiments could possibly have. It appears that the two approaches complement each other in the common effort to understand the mechanisms and rates of *in vivo* conformation changes of nucleic acids.

Acknowledgments

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Supplementary Material Available

The computer programs and the detailed description of the model used in these calculations will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $16.00 for photocopy or $2.00 for microfiche, referring to code number BIO-74-2915.

References

Craig, M. E., Crothers, D. M., and Doty, P. (1971), *J. Mol. Biol. 62,* 383.

Davidson, N., Widholm, J., Nandi, U. S., Jensen, R., Olivera, B. M., and Wang, J. C. (1965), *Proc. Nat. Acad. Sci. U. S. 53,* 111.

Davies, D. R., and Baldwin, R. L. (1963), *J. Mol. Biol. 6,* 251.

Hickey, T. M. (1972), Ph.D. Dissertation, University of Delaware.

Hickey, T. M., and Hamori, E. (1971), *J. Mol. Biol. 57,* 359.

Hickey, T. M., and Hamori, E. (1972), *Biochemistry 11,* 2327.

Inman, R. B., and Baldwin, R. L. (1962), *J. Mol. Biol. 5,* 172.

Inman, R. B., and Baldwin, R. L. (1964), *J. Mol. Biol. 8,* 452.

Laskowski, M., Sr. (1972), *Progr. Nucl. Acid Res. Mol. Biol. 12,* 161.

Pörschke, D., and Eigen, M. (1971), *J. Mol. Biol. 62,* 361.

Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., and Kornberg, A. (1960), *J. Biol. Chem. 235,* 3242.

Scheffler, I. E., Elson, E. L., and Baldwin, R. L. (1968), *J. Mol. Biol. 36,* 291.

Scheffler, I. E., Elson, E. L., and Baldwin, R. L. (1970), *J. Mol. Biol. 48,* 145.

Senior, M. B., Gorrell, S. L. H., and Hamori, E. (1971), *Biopolymers 10,* 2387.

Spatz, H. Ch., and Baldwin, R. L. (1965), *J. Mol. Biol. 11,* 213.

Sueoka, N. (1961), *J. Mol. Biol. 3,* 31.