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Kinetic Investigation of Unfolding and Partial Refolding of a Crab Satellite $(dA\text{-}dT)_n$†

Eugene Hamori,* Takayoshi Iio,‡ Marilyn B. Senior,§ and Peter L. Gutierrez#

ABSTRACT: Crab $(dA\text{-}dT)_n$ was isolated from the testes of *Cancer borealis* by a procedure involving separation of DNA and segregation of the satellite fraction by Hg^{2+} binding/Cs_2SO_4 density gradient ultracentrifugation. The titration of crab $(dA\text{-}dT)_n$ samples at 10° indicated a sharp absorbance change at pH 11.98 in agreement with the pH_m value observed for synthetic poly(dA-dT) under identical conditions. The reversal of the titration, however, resulted only in about 50% recovery of the original absorbance (at 260 nm) in marked contrast to the complete reversibility of the synthetic material. pH-jump experiments were carried out for the purpose of characterizing the rates and mechanisms of conformational transitions brought about by changes in the solution environment. It was found that the disintegration of the putative native structure of crab $(dA\text{-}dT)_n$ starts with a very fast reaction (occurring within the 6-msec deadtime of the instrument and comprising 65% of the total absorbance change) and it is completed via a slower first-order reaction (k = 66 sec^{-1}). It is postulated that the first process is due to the rapid untwisting of end regions and, perhaps, some short hairpin-like helical branches present on the macromolecules. The second reaction is believed to be the end-to-end type unwinding of the double-helical backbone of crab $(dA\text{-}dT)_n$. In the presence of low concentration (3 μg/ml) of Hg^{2+} ions the overall rate of disintegration process decreased drastically. pH jumps from pH values above pH_m to values below were used to study the rates of absorbance changes corresponding to the refolding of the strands of denatured crab $(dA\text{-}dT)_n$. A concentration independent process consisting of two phases was observed. The first phase was a gradual nonexponential process spanning the first second of the reaction, and the other, a very slow first-order process characterized by the rate constant value of 0.053 sec^{-1}. It is proposed that the first part of the process (involving about 24% of nucleotide residues) is an intramolecular formation of helical hairpins (frequently interrupted by mismatching bases) and the second part is a manifestation of some association of the extant unpaired bases during the folding of the branched structure. Refolded crab $(dA\text{-}dT)_n$ samples when subjected again to pH > pH_m in the stopped-flow apparatus displayed not the disintegration pattern of the native crab $(dA\text{-}dT)_n$ but rather that of synthetic poly(dA-dT). The marked facility of crab $(dA\text{-}dT)_n$ macromolecules for rapid conformational transitions induced by slight changes in the solution environment might be relevant to the biological function of this DNA.

Crab $(dA\text{-}dT)_n$ is the general name for various light satellite DNA preparations isolated from crab species (Sueoka, 1961; Widholm and Bonner, 1966; Pochon et al., 1966; Skinner, 1967; Skinner et al., 1970; Skinner and Kerr, 1971; Davidson et al., 1965; Brzezinski et al., 1969; Laskowski, 1972; Sabeur et al., 1969; Ehrlich et al., 1973). The distinguishing feature of these DNAs is their unusual primary structure which is almost entirely a strictly alternating sequence of A[1] and T nucleotide residues (Laskowski, 1972). Base composition and nearest neighbor frequency studies have indicated (Swartz et al., 1962; Laskowski, 1972) that in crab $(dA\text{-}dT)_n$ approximately 93% of the bases are alternating A and T, 4% irregularly occurring A and T, and 3% G and C, with moderate variations among different crab species. Whether the deviations from the al-

† From the Department of Biochemistry, School of Medicine, Tulane University, New Orleans, Louisiana 70112. *Received February 21, 1975.* 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 National Science Foundation (GB-40552). Part of this work was completed at the Department of Chemistry, University of Delaware, Newark, Del.

‡ Present address: Department of Physics, Faculty of Science, Nagoya University, Nagoya, Japan.
§ Present address: Biology Division, Oak Ridge National Laboratories, Oak Ridge, Tenn. 37830.
# Present address: Department of Radiology, Medical College of Wisconsin, Milwaukee, Wis. 53226.

[1] Abbreviations used are: A, deoxyadenylic acid residue or adenine base; T, deoxythymidylic acid residue or thymine base; G, deoxyguanylic acid residue; C, deoxycytidylic acid residue; SSC, 0.15 *M* NaCl-0.015 *M* sodium citrate buffer; 0.1 SSC, 0.015 *M* NaCl-0.0015 *M* sodium citrate buffer; A_{260}, absorbancy at 260 nm; EDTA, ethylenediaminetetraacetic acid; pH_i and pH_f, the initial and final pH values in a pH jump experiment.

ternating A-T structure occur strictly randomly or represent some long-range repeating pattern is not known at the present time. In view of the peculiar species-specific nature of satellite DNAs (Comings, 1972; Rae, 1972; Flamm et al., 1969), however, these "irregularities" most likely represent important structural characteristics. According to the electron micrographs of Davidson et al. (1965) the long fragments of crab (dA-dT)$_n$ isolated in the native form from *Cancer antennarius* exist in the normal unbranched conformation in contrast to the branched star-like conformation of the synthetic poly(dA-dT).[2] Similarly to other repetitive DNAs crab (dA-dT)$_n$ fractions fragmented by sonication renature much more rapidly in a denaturation–renaturation cycle than their respective main-fraction DNAs. It has been generally accepted that crab (dA-dT)$_n$ is not transcribed into protein, ribosomal RNA components, tRNA, etc., and therefore (together with other satellite DNAs) it might be considered as "nongenal" DNA. The possible biological role of satellite DNAs has been discussed in several review articles (Southern, 1974; Comings, 1972; Yunis and Yasmineh, 1971; Rae, 1972).

The purpose of the present investigation was to study the rates and mechanisms of crab (dA-dT)$_n$ conformation changes produced by sudden variations of the pH of the solutions. Specifically we wanted to compare crab (dA-dT)$_n$ kinetic results to those obtained on poly(dA-dT) in a recent study completed in our laboratory (Iio et al., 1974) and interpret differences in terms of the known primary structure and conformational differences between the two nucleic acids.

Experimental Section

Materials. The marine crabs *Cancer borealis* were obtained from the Marine Biological Laboratory, Woods Hole, Mass. Crystalline pancreatic RNase, T_1 RNase, and Pronase (a nonspecific protease from *Streptomyces griseus*) were purchased from Sigma Chemical Co., St. Louis, Mo. All chemicals used were reagent grade, and all solutions were prepared with doubly distilled water.

Isolation of Whole Crab DNA. The isolation procedure was essentially the technique of Marmur (1961) as modified by Baranowska et al. (1968) and Brzezinski et al. (1969); however, to minimize enzymatic degradation of the DNA, fresh tissue was used. The vas deferens and testes from 120 live male *C. borealis* were removed, suspended in a cold (~4°) solution of 0.1 *M* NaCl–0.1 *M* EDTA (pH 7.49), and homogenized. Sodium dodecyl sulfate (25% in 20% ethanol) was added to a final concentration of 3%, and the mixture was stirred at room temperature for 2.5 hr. NaCl was added to a final concentration of 1.1 *M*, and the solution was stirred an additional 0.5 hr. The mixture was centrifuged in the cold for 30 min at 5500 rpm in a Sorvall S-1 centrifuge, and the supernatant liquid was added to an equal volume of chloroform–isoamyl alcohol mixture (24:1, v/v). The liquid was centrifuged in a Sorvall R-2 centrifuge for 7 min at 7000 rpm at 0°, and the top aqueous layer removed. An equal volume of chloroform–isoamyl alcohol mixture was added to the supernatant, the solution was shaken gently for 10 min, and the centrifugation was repeated. NaCl was added to a final concentration of 4 *M*, and the deproteinization procedure was repeated until only traces of protein were visible at the interface. The aqueous solution was chilled, diluted with 1 vol of water, and added in 100-ml portions to 200-ml volumes of cold 95% ethanol. The fibrous DNA–RNA–histone complex was spooled on swirling glass rods, pressed to remove excess ethanol, and dissolved in 0.15 *M* NaCl–0.015 *M* sodium citrate (SSC)[1] solution by stirring overnight in the cold. Some denatured protein which was precipitated along with the DNA complex did not redissolve in the SSC solution, and the solution was centrifuged to remove this precipitate. Pronase was added to the supernatant to a concentration of 250 μg/ml; the mixture was incubated at 40° for 2 hr, deproteinized with chloroform–isoamyl alcohol 3 times, and precipitated with ethanol. The spooled material was dissolved in cold 0.015 *M* NaCl–0.0015 *M* sodium citrate buffer. In order to remove contaminating RNA the solution was treated with 100 μg/ml of pancreatic RNase (which had been heated to 80° for 10 min to destroy DNase activity) and 1000 units/ml of T_1 RNase. The enzymes were allowed to act for 2 hr at 40°, Pronase was added (100 μg/ml), and the mixture incubated an additional 2 hr to remove the nucleases and any traces of other proteins. The system was deproteinized with chloroform–isoamyl alcohol 3 times and precipitated with ethanol 3 times. The fibrous gel was dissolved in 0.1 SSC and frozen. This material is referred to in this paper as whole crab DNA.

Separation of Crab (dA-dT)$_n$ and Main-Fraction DNA. The Hg^{2+} binding/Cs_2SO_4 density gradient centrifugation method was used to separate the two crab DNA components (Davidson et al., 1965; Klett and Smith, 1967). Two milliliters of whole crab DNA solution (approximately 0.5 mg/ml) was dialyzed vs. 200 ml of 5×10^{-3} *M*, pH 9.22, sodium borate buffer for 24 hr at 4° with 3 changes of buffer. (Since the Hg^{2+} binding technique requires the absence of Cl^-, the sodium borate buffer was used as the solvent in the preparative ultracentrifugation work.) To 1.75 ml of the DNA solutions, 4.20 ml of Cs_2SO_4 (1 g/ml) and 0.46 ml of 5×10^{-4} *M* $HgCl_2$ (both in sodium borate buffer) were added. The solution was adjusted to the proper density by setting its refractive index to 1.3730 by addition of 5×10^{-3} *M* sodium borate buffer (Klett and Smith, 1967). Two-milliliter portions of this solution were added to cellulose nitrate centrifuge tubes, and 3 ml of paraffin oil was layered on top of each tube. The solutions were spun at 31,000 rpm in a preparative ultracentrifuge at 19° for 40 hr. The centrifuge was allowed to stop without braking and 30 fractions (7 drops each) were collected from the bottoms of each tube. The fractions were diluted with 1 ml of water, and the A_{260} values read on a spectrophotometer were plotted on a histogram. The contents of tubes corresponding respectively to the main and satellite fractions were pooled and dialyzed at 4° against the following succession of solutions: SSC, 0.1 SSC (both containing 10 m*M* EDTA), and 5 m*M* NaCl. The purified crab (dA-dT)$_n$ and main-fraction DNA were frozen in 5 m*M* NaCl for storage.

Solutions. Crab (dA-dT)$_n$ solutions were prepared either by dialyzing aliquots of stock solutions containing purified crab (dA-dT)$_n$ against the desired buffer or simply by adding the stock solution to the buffer. During the unwinding studies special care was taken to avoid even the momentary exposure of crab (dA-dT)$_n$ to a pH milieu close to the melting pH (pH_m) of the system. In some cases it was necessary to concentrate crab (dA-dT)$_n$ solutions prior to their use in the experiments. The procedure generally used involved the gentle stirring of the liquid at room temperature in a small erlenmeyer flask for about a day, while filtered, CO_2-free,

[2] This synthetic DNA was referred to as poly[d(A-T)] in our previous publications.

dry air was blown over the surface of the solution.

Preparation of Denatured Crab $(dA\text{-}dT)_n$. Eight milliliters of native crab $(dA\text{-}dT)_n$ solution containing 25 m*M* Na^+ and 4 m*M* PO_4^{3-} (pH 7.0, $A_{260} = 0.1$) was heated to 50° and was kept overnight at this temperature. In order to ensure the complete separation of the two strands of DNA molecules 0.12 ml of 1 *N* NaOH was added to the system (pH ~12.3). The solution was then immediately cooled to 10° and the pH readjusted to 7.0. Using the concentration procedure described above the final volume was reduced to 3 ml, and the solution was dialyzed against 13 m*M* NaCl and 4 m*M* Na_3PO_4.

Preparation of Crab $(dA\text{-}dT)_n$ Solutions Containing Hg^{2+} Ions. In order to test the effect of Hg^{2+} ions on the kinetic properties of crab $(dA\text{-}dT)_n$ a sample was prepared by a purification process in which the dialysis times were shortened and EDTA was omitted from solutions. In this particular preparation the residual concentration of Hg^{2+} was 114 μM (DNA concentration, 41 μg/ml) in contrast to the fully dialyzed sample of 1.0 μM Hg^{2+} concentration (DNA concentration, 12.9 μg/ml). The mercury analyses were performed by a flameless atomic absorption method at Gulf South Research Institute, New Orleans, La.

pH Determinations. The pH of the nucleic acid solutions was measured with a Beckman Expandomatic pH meter (Model SS-2) which was standardized at 10.0° before each series of determinations at pH 7.06 with a standard buffer (Corning, 477070) and at 13.00 with a saturated $Ca(OH)_2$ solution. The latter standard was prepared according to the laboratory procedure previously described (Senior et al., 1971). The pH adjustment of the solutions was carried out in a glove box equipped with iris type ports. In order to exclude CO_2 the box was continuously flushed with air conducted through a column of NaOH pellets. During pH measurements the temperature of the buffers and polymer solutions was maintained at 10 ± 0.1° by circulating water through a double-walled titration vessel from a temperature controlled circulator bath. The pH of the solutions was adjusted by adding small amounts of 1 *N* NaOH or HCl from a microsyringe.

Determination of the pH Melting Curves. A mixture of 2.5 ml of 16 m*M* Na_3PO_4 and 0.125 ml of 1 *N* NaCl was diluted with water to 9 ml total volume and the pH was adjusted to 11.40 by the addition of 1 *N* HCl. One milliliter of stock crab $(dA\text{-}dT)_n$ solution containing about 10 μg of DNA in 5 m*M* NaCl was added to this buffer and the solution was placed into a titration vessel thermostated at 10°. The pH of the system was raised in small increments by additions of 1 *N* NaOH. After each pH adjustment an aliquot of the solution was placed in a spectrophotometer (Coleman, Model 46), also thermostated at 10°, and its A_{260} value determined.

Sedimentation Velocity Measurements. (A) Crab $(dA\text{-}dT)_n$; Neutral Conditions. A 1.3-ml sample of the crab $(dA\text{-}dT)_n$ solution was dialyzed against 450 ml of 0.1 *N* NaCl–0.05 *M* phosphate buffer (pH 7.0) for 24 hr at 4° with 2 changes of buffer; according to the A_{260} value of the solution the concentration of nucleic acid was 6 μg/ml after dialysis. The solution was spun at 42,040 rpm in the analytical ultracentrifuge at 20° in a 12-mm single-sector cell. The ultraviolet (uv)-absorption photographs of the sedimentation velocity experiments were scanned with a Canalco Model K/86 spectrodensitometer (Canalco, Inc., Rockville, Md.). The value of the sedimentation coefficient, *s*, was obtained according to the procedure of Schumaker and Schachman (1957).

(B) Crab $(dA\text{-}dT)_n$; Alkaline Conditions. A 0.02-ml sample of crab $(dA\text{-}dT)_n$ solution ($A_{260} = 0.8$) 10 m*M* in NaCl and 20 m*M* in NaOH was mixed with 0.65 ml of a solution 0.9 *M* in NaCl and 0.1 *M* in NaOH. The sample was placed in the ultracentrifuge cell and treated as above.

(C) Main-Fraction DNA. The nucleic acid sample in 10 m*M* NaCl (80 μg of DNA/ml) was layered on the top of 1 *N* NaCl solution in a cell equipped with a band-forming centerpiece (Beckman, No. 331340) according to the procedure of Studier (1965). The solution was spun at 42,040 rpm at 20° and the uv-absorption photographs were scanned as described above. The sedimentation constant was determined according to Bauer and Vinograd (1971) and Studier (1965).

Kinetic Measurements. The rate of conformation change of nucleic acid samples was measured in a Durrum Gibson stopped-flow spectrophotometer by experimental procedures analogous to those described in detail previously (Hickey and Hamori, 1971, 1972; Iio et al., 1974). For all experiments the temperature of the solutions in the stopped-flow apparatus was maintained at 10 ± 0.05°. The conformation change involving the unfolding of the native double-helical structure of crab $(dA\text{-}dT)_n$ was initiated by a sudden increase in the pH of the solutions. In order to bring about these pH jumps the nucleic acid samples in 4 m*M* Na_3PO_4 and 13 m*M* NaCl (at pH values lower than transition pH) were mixed in the stopped-flow instrument with an alkaline solution containing 25 m*M* Na^+, 4 m*M* PO_4^{3-}, and Cl^-. The pH of the latter solutions was adjusted in a manner such that, after mixing, the pH of the resultant solution was at a value favoring the unfolded (random coil) conformation of the DNA molecules. The conformation change involving the partial refolding of crab $(dA\text{-}dT)_n$ molecules (from an unfolded state at pH > pH_m) was initiated in an analogous manner by a pH jump in the opposite direction. The dead time of the stopped-flow apparatus was determined to be 3 msec at 20° and 6 msec at 10°, by using $Fe(NO_3)_3$ and KCNS solutions in a procedure previously described (Hickey, 1972). For the determination of rate constants the oscilloscope traces were photographed and replotted on semilogarithmic graphs.

Results

Characterization of Crab $(dA\text{-}dT)_n$. In the ultraviolet spectrum of the isolated whole crab DNA the 280/260 and 230/260 absorbance ratios were found to be 0.523 and 0.430, respectively. On the melting curve of this DNA sample in SSC two transitions could be seen. The first, corresponding to the melting of satellite DNA, occurred at approximately 65°. The second, the transition for the main DNA moiety, was around 85° in this solvent and it was broad (Senior, 1972). Literature values for the melting temperature of *C. borealis* crab $(dA\text{-}dT)_n$ in SSC are 64.2° (Sueoka and Chang, 1962), 65.2° (Smith, 1964), 65.7° (Widholm and Bonner, 1966), and 66° (Waldvogel and Swartz, 1971).

In a typical separation experiment using Hg^{2+} binding/Cs_2SO_4 density gradient ultracentrifugation two peaks were obtained. The first was identifed as "main-fraction DNA" and the second as crab $(dA\text{-}dT)_n$. The latter fraction accounted for 27% of the total absorbance of the sample at 260 nm. This was in agreement with the previously reported satellite DNA content of 30% of *C. borealis* DNA (Smith, 1964). The λ_{max} for the main fraction peak occurred at 259

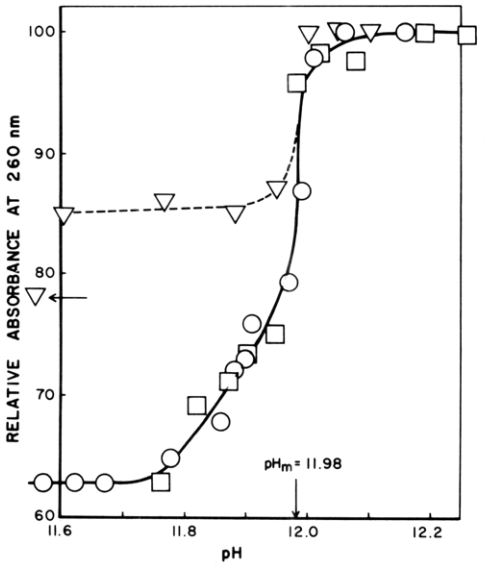

FIGURE 1: pH melting curve of crab d(A-T)$_n$. The symbols ○ and ▽ correspond respectively to data taken in one experiment during the increase and subsequent (after a 5-min waiting period) decrease of pH. The arrow indicates a measurement taken at pH 11.4 after 24 hr. The symbol □ designates data obtained in a separate experiment involving only the increase in pH. In all experiments the temperature was 10°, the concentration of PO_4^{3-} 4 m*M*, and the initial concentration of Na^+ 25 m*M*.

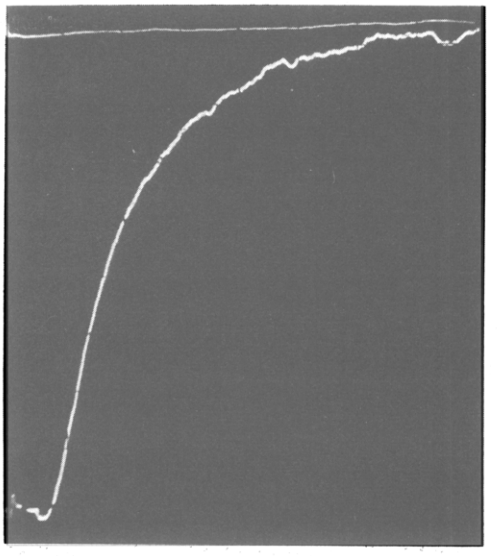

FIGURE 2: Oscilloscope record of the rapid unwinding of double-helical crab d(A-T)$_n$: ΔpH 11.38 → 12.08; 25 m*M* PO_4^{3-}; 4 m*M* Na^+; 10°; 8.8 μg of DNA/ml. The horizontal scale is time (80 msec/full scale) and the vertical scale is absorbance at 260 nm.

nm, and that for the satellite DNA at 263 nm. This shift in λ_{max} was in accord with the very high A-T content of crab satellite DNA. The temperature melting curve of the isolated crab (dA-dT)$_n$ in 0.1 SSC indicated a single very sharp increase in absorbance (λ 260 nm) at 47°. Upon cooling the absorbance returned near to its original value. (The hypochromicity observed on cooling was about 95% of the hyperchromicity observed on heating.)

Figure 1 illustrates the absorbance change of the isolated crab (dA-dT)$_n$ at λ 260 nm upon variation of the pH of the solution. The irreversibility of the titration curve seen is an indication that the DNA is in a native state. The slight premelting of the sample in the pH range of 11.8–11.9 appears to be an artifact. During the titration in this particular pH region the absorbance values first increased and then decreased after each incremental addition of base. This suggests that the momentary local pH increase after each base addition caused the unfolding of some DNA molecules which subsequently refolded but, due to the irreversibility of the process, only incompletely. Pochon et al. (1966) reported very similar results for their *Cancer pagurus* crab (dA-dT)$_n$ preparation. Their pH$_m$ value (10.75), however, is less than ours (11.98) as would be expected in view of the higher temperature (room temperature) and high salt concentration (0.15 *M* NaCl) used by these workers. The titration curve (not shown) of crab (dA-dT)$_n$ solutions containing mercury ions indicated a significantly reduced total hyperchromicity (18% vs. 37% in terms of the scale of Figure 1) and an upward shift of the transition to a pH value around 12.3. Only 40% of the absorbance change was reversible upon lowering the pH of the solution.

Ultracentrifugation experiments in neutral solutions indicated an $s_{20,w}$ value of 16.4 for the main-fraction DNA and values of 8.7 and 9.7, respectively, for the two crab (dA-dT)$_n$ samples measured. The latter of the crab (dA-dT)$_n$ samples was also tested under alkaline conditions yielding an $s_{20,w}$ value of 14.2. Further details of the characterization of crab (dA-dT)$_n$ samples have been described elsewhere (Senior, 1972).

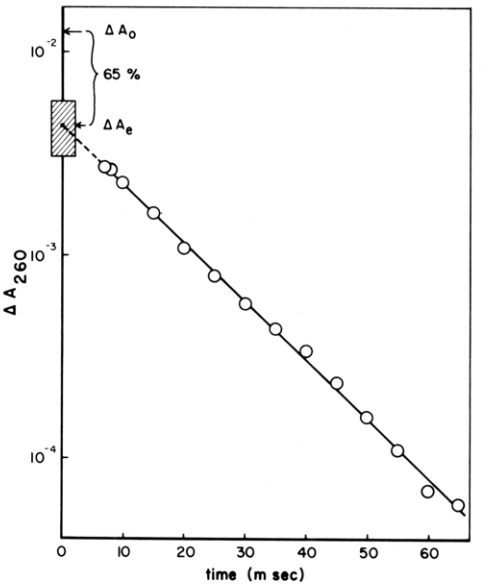

FIGURE 3: First-order plot of the process shown in Figure 2. The theoretical initial value of ΔA_{260} corresponding to the absorbance of solution at the instant of mixing is designated by ΔA_0; it was obtained by an independent measurement outside the stopped-flow instrument. The value of ΔA_{260} obtained by extrapolating the kinetic data to zero time is designated by ΔA_e. The shaded area represents the experimental error involved in estimating the fast unrecordable part of the reaction. The first-order rate constant calculated from this plot is $k_u = 66$ sec^{-1}.

Kinetic Studies. (A) Measurements of Unwinding Rates. A typical result of a stopped-flow experiment on crab (dA-dT)$_n$ is shown in Figure 2. It is a record of the absorbance change at 260 nm following the sudden rise of pH, and it indicates the time course of the disintegration of the double helical structure of native crab (dA-dT)$_n$. It can be seen from the semilogarithmic plot of the same reaction (Figure 3) that the single first-order process observed cannot be extrapolated back to the initial absorbance of the system at the beginning of the conformation change. This indicates that a separate fast process must precede the reaction actu-

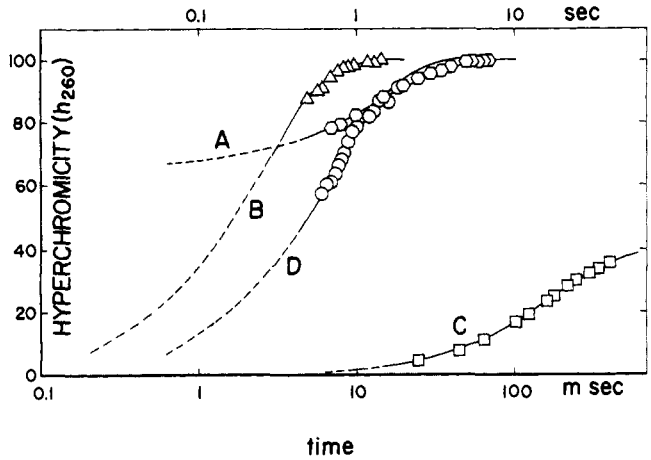

FIGURE 4: Representation of the unwinding process of various nucleic acid samples on a logarithmic time scale. The formula used for calculating the ordinate value h is given in the text: (A) data of experiment shown in Figures 2 and 3; (B) synthetic poly[d(A-T)]; ΔpH, 11.53 → 12.16; DNA concentration, 12.5 μg/ml (from Iio et al., 1974); (C) crab d(A-T)$_n$ containing Hg^{2+} ions (3 μg/ml); ΔpH, 11.82 → 12.18; DNA concentration, 5.1 μg/ml (the time scale of this curve is on the *top* of the diagram); (D) crab d(A-T)$_n$ which had been denatured and subsequently (partially) renatured (see text) prior to the unwinding experiment; ΔpH, 11.62 → 12.14; DNA concentration, 13.0 μg/ml. The lines drawn in this graph are those *calculated* from the corresponding semilogarithmic plots (e.g., Figure 3). Note that the excessive length of extrapolated regions (dotted lines) is due to the peculiarity of the logarithmic time scale used.

ally observed. Due to the dead time of the stopped-flow apparatus this fast initial phase, constituting about 65% of the total absorbance change, could not be directly recorded. (The relatively large error associated with this value (see Figure 3) was due to uncertainties both in the determination of zero time and in the comparison of absorbances determined in two separate instruments.) It was also established that the conformation change observed was not followed by slower reactions. In Figure 4, curve A, the progress of the same conformation change is illustrated on a logarithmic time scale.[3] The ordinate (h) of this plot is the percentage of the (final) hyperchromicity reached during the course of the reaction. It was calculated using the equation:

$$h_t = \frac{(A_t - A_i)}{(A_f - A_i)} \times 100 \qquad (1)$$

where A_t is the absorbance of the solution (at 260 nm) recorded at time t, A_i is the absorbance determined for $t = 0$ (i.e., half of the absorbance of the sample in the stopped-flow instrument prior to the 1:1 dilution by the base solution), and A_f is the final absorbance. It can be seen in Figure 4, curve A, also, that the reaction seen does not extrapolate back to the initial absorbance of the system.

In a series of experiments in which the initial pH of the pH jump varied from 7.4 to 11.4 and the final pH from 12.0 to 12.4 the rate constant of the unwinding reaction was found to be in the range between 40 and 100 sec^{-1}. Although it was established that raising the final pH value above the range of 12.2–12.4 increased the rate of disintegration of double helical structure, no obvious relationship could be observed between the initial pH of the jump and the rate of the reaction.

[3] In such a representation a first-order process appears as a sigmoidal curve whose inflection point is at $t = 1/k$.

The semilogarithmic plot (not shown) of the unwinding reaction of the Hg^{2+}-containing crab (dA-dT)$_n$ sample also indicated a first-order process but, in contrast to the pure crab (dA-dT)$_n$ system, the observed reaction started from the (theoretical) initial absorbance of the system. The unwinding rate was much slower (k_u = 0.053 sec^{-1}) than that of pure crab (dA-dT)$_n$ (k_u = 66 sec^{-1}) and the total hypochromicity displayed was small (18%). Figure 4, curve C, is the representation of this reaction on a logarithmic time scale. In the calculation of the ordinate values for this curve it was assumed that the reduced hypochromicity observed was entirely due to the limited unfolding of the Hg^{2+}-complexed double helical structure and, therefore, the value of A_f used (eq 1) was not the value actually observed but a hypothetical one which the solution would have displayed if it had completely unfolded similar to the Hg^{2+}-free sample. Figure 4, curve D, is the unwinding curve of a crab (dA-dT)$_n$ sample which was first denatured at high pH (see Experimental Section) and subsequently brought back to neutral pH prior to the kinetic experiment. The semilogarithmic plot of the reaction (not shown) indicated a single process (k_u = 140 sec^{-1}) extrapolating back to the initial absorbance of the system. Curve B of Figure 4 is the unwinding curve of a synthetic (dA-dT)$_n$ sample (no. 5, $s_{20,w}$ = 8.68) as determined by our previous investigation (Iio et al., 1974). The rate constant of the single exponential process observed is 407 sec^{-1}.

A small number of experiments were carried out in order to characterize the unwinding rate of main-fraction crab DNA. For pH jumps comparable to those of crab (dA-dT)$_n$ experiments (e.g., 11.6–12.2) the unwinding reaction observed originated from the initial absorbance of the system (i.e., there was no fast reaction which would have been unobservable because of instrumental limitations) and proceeded via a gradually decelerating process lacking phases identifiable as first-order reactions. Typically, about 55% of the total absorbance change was completed within the first 100 msec, about 80% within 10 sec, and about 97% within 100 sec.

(B) Measurements of the Rate of Refolding of Crab (dA-dT)$_n$. These experiments involved the sudden *lowering* of the pH of crab (dA-dT)$_n$ solutions from a value above the transition pH to a value below. Under these conditions the nucleic acid molecules undergo a random-coil to (partial) double-helix conformation change. Figure 5 (curve A) and Figure 6 represent typical results of such rewinding experiments. (See captions for details of experimental conditions.) The evaluation of several sets of such data taken under different experimental conditions indicated the following. The partial renaturation reaction actually recorded was not preceded by an unobservable fast process. The total absorbance change exhibited was around 20% in agreement with the partial reversibility of the system displayed during the static pH melting experiments (Figure 1). The process observed had two phases. The first phase spanned the first 10 sec of the reaction and it was not an exponential process; the second phase was a very slow reaction progressing in the time range of minutes. The latter part of the conformation change was clearly a first-order reaction (Figure 6). The rates observed showed no significant variations with nucleic acid concentration in the range of 0.9–10.0 μg/ml.

Discussion

Using the empirical relationship suggested by Studier (1965) for the molecular weight of a native DNA ($s_{20,w}$ =

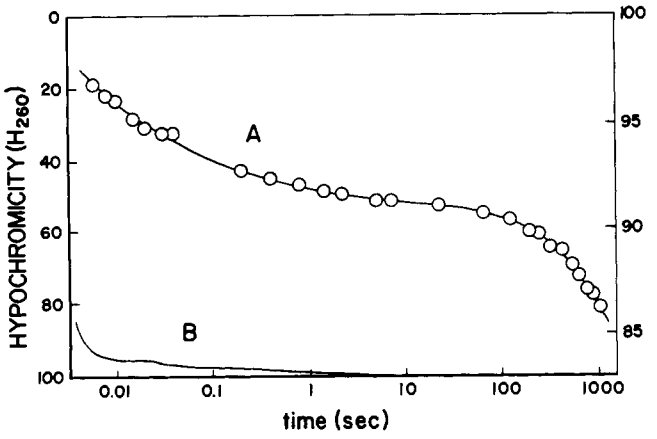

FIGURE 5: Representation of the pH change induced refolding of nucleic acid samples on a logarithmic time scale. The ordinate values for the scale on the left side were calculated according to eq 1 given in the text: (A) crab d(A-T)$_n$; ΔpH, 12.34 → 11.63; 25 mM PO_4^{3-}; 4 mM Na^+; 10°; 8.5 μg of DNA/ml; (B) synthetic poly[d(A-T)], the results of Hickey and Hamori (1972), the experimental conditions were the same as those of curve A. The scale on the right side is for curve A only; it is the relative absorbance at 260 nm (same as the scale of Figure 1). The values of the sedimentation constants ($s_{20,w}$) of the DNA samples used were 9.7 and 9.2 for curves A and B, respectively.

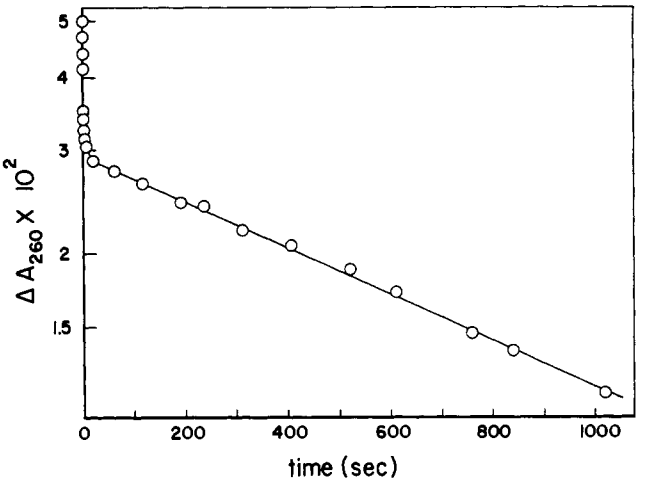

FIGURE 6: First-order plot of the process shown in Figure 5A. The slope of the straight-line portion of the curve yields $k_f = 9.3 \times 10^{-4}$ sec^{-1} for the second phase of the rewinding reaction.

$0.0882M^{0.346}$) we estimated that the molecular weight of the main-fraction DNA sample was 3.6×10^6 and those of the two crab (dA-dT)$_n$ fractions tested were 5.8×10^5 and 7.9×10^5, respectively. From the alkaline sedimentation results of the latter crab (dA-dT)$_n$ sample we calculated in a similar manner ($s_{20,w} = 0.0528M^{0.40}$) a molecular weight of 11.9×10^5. These results indicate that crab (dA-dT)$_n$ did not degrade into low molecular weight fragments and did not incur single-strand breaks during the isolation procedure. (Such damage was observed in another crab (dA-dT)$_n$ preparation described by Widholm and Bonner (1966).)

The comparison of the pH melting curve of crab (dA-dT)$_n$ (Figure 1) to that of synthetic (dA-dT)$_n$ (Hickey and Hamori, 1972) indicates an identical pH_m value (11.98) for both nucleic acid samples. This means, of course, that the presence of a small amount (3%) of G·C base pairs does not provide the helical crab (dA-dT)$_n$ structure a significantly enhanced stability at high pH environment. Both the slight premelting of crab (dA-dT)$_n$ (see Discussion section above) and the limited (~50%) reversibility of the titration curve are indications for a native DNA structure which is unable to re-form completely when the denaturing conditions are removed. Similarly to our findings, Davidson et al. (1965) reported that the *thermal* denaturation of their native crab (dA-dT)$_n$ sample was 93% reversible. Pochon et al. (1966) found that their crab (dA-dT)$_n$ prepared by a chromatographic technique (i.e., not entirely native) displayed an almost completely reversible thermal denaturation but only a 77% reversible (alkaline) pH denaturation. These observations suggest that denaturation of this particular DNA at high pH values is more thorough and, therefore, less reversible than that normally achieved by temperature melting.

The pH change induced unwinding of double helical crab (dA-dT)$_n$ shows an initial very fast process whose time range falls beyond the resolution of our stopped-flow instrument (Figure 3). Since such fast initial reaction was not observed either with poly(dA-dT) or with previously denatured crab (dA-dT)$_n$ this process appears to be characteristic to the native double-helical structure of crab (dA-dT)$_n$ as isolated by our purification procedure. Hairpin structures formed at the frayed ends or at partially unwound inner sections of the extended double helix of native crab (dA-dT)$_n$ would be expected to unwind very fast[4] and their presence could explain the observed rapid initial phase of the denaturation process. The very fast reaction is followed by a slower process progressing in the 10–50-msec time range (Figure 3 and Figure 4, curve A). It is significantly slower than the rate of unwinding of poly(dA-dT) measured under comparable conditions (Figure 4, curve B) and it could be related to the end-to-end type unwinding of the native double helical structure. It is to be noted that the unwinding rates of both the synthetic (dA-dT)$_n$ and the unfolded–refolded crab (dA-dT)$_n$ are much slower than the very fast phase of the denaturation process of native crab (dA-dT)$_n$. The explanation might be that while all these systems contain hairpin loops, these branches are far apart on our native crab (dA-dT)$_n$ molecule but they are crowded together in the irregularly folded conformations characteristic to synthetic poly(dA-dT) and unfolded–refolded crab (dA-dT)$_n$. Thus, it is expected that the unwinding rates of the latter samples would be significantly hindered by steric interference among the untwisting helical branches.

The data shown in Figure 4, curve C, indicate that the presence of Hg^{2+} ions will drastically retard the unwinding rate of native crab (dA-dT)$_n$. This result together with the observed decrease of hyperchromicity of the Hg^{2+}-containing samples suggest a strong DNA–Hg^{2+} complex in which the two DNA strands (of not necessarily the same conformation as in Hg^{2+}-free samples) separate rather reluctantly even after the destabilization of the helix.

When considering the relatively slow unwinding rates of some other DNA samples investigated (Crothers, 1964) and that of main-fraction crab DNA (see above) the overall unwinding rate of crab (dA-dT)$_n$ appears to be exceedingly fast. The facility of this satellite DNA for rapid conformational changes might have biological significance (see more below).

It was established in our previous studies (Iio et al., 1974) that the unwinding rate of synthetic (dA-dT)$_n$ increases when the final pH value of the pH jump is in-

[4] The untwisting of these hairpin structures generates very little hydrodynamic resistance.

creased. This observation was explained by the fact that at higher pH values the number of negative charges present on the molecule increases and the resulting stronger electrostatic repulsion enhances the separation rates of the two strands of the double helix. In our limited pH-dependence study of crab $(dA\text{-}dT)_n$ a similar effect was observed presumably due to the same cause as postulated for the synthetic sample. For poly(dA-dT) the manifested effect of the initial pH of the pH jump on the unwinding rate was due to the fact that the initial conformation of the macromolecules could vary according to pH_i values (Iio et al., 1974). Our results on crab $(dA\text{-}dT)_n$ indicated no effect of pH_i on the rate of unwinding aside from some spurious results which could be explained by irreversible denaturation due to the local pH effects.

We shall proceed to discuss now our helix rewinding experiments in which the pH was *decreased* from a value above pH_m to a value below and this change brought about a decrease in the absorbance of crab $(dA\text{-}dT)_n$ samples. The important observation that the rates measured were independent of the nucleic acid concentration of the solutions must mean that the process monitored did not involve separated single strands but rather it was an intramolecular process involving the folding back of individual strands into a branched structure of several helical hairpins. The model of helix refolding proposed in our previous publication (Hickey and Hamori, 1972) can be invoked to explain the large differences observed between the rates of refolding of synthetic $(dA\text{-}dT)_n$ and crab $(dA\text{-}dT)_n$ (Figure 5). According to this model the very fast initial rate of the synthetic material (the missing initial portion of curve B) is associated with the rapid twisting of hairpin-like branches formed on the individual single strands of the separated DNA chains. Since in poly(dA-dT) all the aligned bases match (subsequent to the formation of a nucleating loop) the hairpin-like helical structures will propagate unhindered (at sufficiently low pH_f) until two growing branches converge. For this reason a large fraction (more than 90% in Figure 5, curve B) of the conformation change proceeds via this effective mechanism. In contrast to synthetic $(dA\text{-}dT)_n$, crab $(dA\text{-}dT)_n$ contains 4% irregularly occurring A·T bases and 3% G·C bases in addition to the (93%) strictly alternating A·T sequences. Although the exact distribution of bases other than alternating A·T has not been determined yet, several studies suggest a seemingly random distribution of G·C base pairs with very little clustering (Laskowski, 1972). It is easy to see that with this type of base sequence the growth of hairpin loops developing on the regular ····A-T-A-T-A-T-···· sections of the chain must be constantly interrupted by G and C and irregularly occurring A and T bases. After each such arrest the growth of a certain hairpin can be resumed only when the strands manage to detour the obstacle of the mismatching base. This latter step, however, being commensurate with the nucleation of a new helix, is rather slow and will reduce drastically the overall rate of double helix formation. Although various models could be devised involving an efficient "flipping out" of unfitting bases and thereby allowing the helix growth to continue, our kinetic results clearly show that such a mechanism is not operative under our experimental conditions, and as a result, a relatively small fraction of unmatching bases will have an inordinate effect on the rate of helix formation. A similar arresting effect of methylated bases and intercalating agents has been already demonstrated on the refolding process of poly(dA-dT) (Hickey and Hamori, 1972) and the effect of unmatching base pairs observed on a native DNA in the present study was predictable.

The refolding process of poly(dA-dT) consists of a major and two minor exponential reactions with approximate half-life values of 1 msec, 35 msec, and 3 sec, respectively (Hickey and Hamori, 1972). In contrast, the rewinding curves of crab $(dA\text{-}dT)_n$ display only two processes. One spans the initial 10 sec of the reaction and the other progresses in the time range of minutes (Figure 5). Only the second process follows first-order kinetic behavior (Figure 6). The possibility that either or both reactions reflect inter-chain type double helix formation can be excluded by the observed concentration independence of the rates. We interpret the first process as the stop-and-go type development of double-helical hairpins discussed above. This phase represents about 55% of the total hypochromicity observed upon lowering the pH. The remaining 45% of the process is an extremely slow reaction which could either be associated with a continuation of the growth of helical structure (e.g., by the occasional opening up of small hairpins and subsequent re-formation of a more completely base-paired helical structure) or, alternatively, it could be an absorbance change reflecting not further helix growth but some other type of interaction among the large number (76%, see discussion below) of unpaired bases still extant on the macromolecules. The attractive forces involved among the bases protruding from the helical hairpins of the highly branched structure must be hydrogen bonds and nonbonded interactions (e.g., London forces, dipole–dipole interactions, etc.). These would result in a stabilization of the folded structure of the nucleic acid molecules. Based on the absence of such a phase in the rewinding curve of synthetic $(dA\text{-}dT)_n$ (Figure 5) we prefer to consider the second phase of the refolding reaction of crab $(dA\text{-}dT)_n$ as the interaction of the unpaired bases protruding from hairpin branches rather than a slow improvement of the imperfect base pairing.

Since the scale on the right side of Figure 5 is identical with the ordinate of Figure 1 (in both cases the absorbance of the DNA solution at $pH \gg pH_m$ is taken to be 100) the partial refolding of crab $(dA\text{-}dT)_n$ during back titration (Figure 1) and following a fast pH jump (Figure 5) can be compared. It can be seen that the total hypochromicity observed was 15–22% in the first case and about 17% in the second case. In view of the entirely different conditions the DNA samples were subjected to in the two types of experiments this agreement is good. The inspection of Figure 1 reveals that the complete re-formation of the native structure should be accompanied by a 37% hypochromicity. It follows then that the approximately 9% hypochromicity observed at the fast phase of the rewinding reaction (Figure 5) corresponds to $0.09/0.38 = 24\%$ recovery of the original amount of base pairing of the native structure. This estimation implies that at the end of the fast reaction about 76% of the bases is still unpaired. At the end of the *slow* reaction when the hypochromicity reaches 17% this method of estimation would yield 45% for the recovery of the original helix content. In view of the above discussion, however, this is not a correct calculation since during the slow reaction the adsorbance change is due to the irregular association of extant bases rather than further double helix formation. In other words, we tend to believe that in the refolded state of an average crab $(dA\text{-}dT)_n$ molecule 24% of the bases are paired in double helices and 76% of them are involved in other interactions, less effective in inducing hypochromicity than base pairing inside the double helix.

The reassociation of highly repetitive DNA fractions under conditions favoring intermolecular double helix formation is known to be much faster than that of genal DNA. Our present investigations have shown for crab (dA-dT)$_n$ that even in dilute solutions where an intramolecular process (hairpin formation) is favored about half of the refolding process is completed within a time period of 1 sec. We have also established that the unwinding process of crab (dA-dT)$_n$ is fast when compared to that of main-fraction crab DNA.

It has been suggested that the functioning of DNA in chromosomes could be under the regulatory control of highly repetitious nucleotide sequences distributed along the main DNA chain (Crick, 1971; Paul, 1972). It was proposed, for instance, that interactions with specific regulatory proteins could trigger conformation changes in these strategically located DNA regions and these, in turn, would cause major alterations in the higher order structure of chromatid fibers. Such changes, on the other hand, could be prerequisite for RNA polymerase action. Our findings in this paper are very much in accord with such a postulated role for satellite DNA fragments. It is possible, therefore, that the marked facility of crab satellite (dA-dT)$_n$ for rapid double-helix/random-coil type conformational transition is related to the in vivo functioning of this nucleic acid.

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