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Photochemical Demonstration of Stacked C·C$^+$ Base Pairs in a Novel DNA Secondary Structure†

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Received May 29, 1984

ABSTRACT: The secondary structure of the alternating polydeoxynucleotide sequence poly[d(C-T)] was studied as a function of pH by ultraviolet absorbance and circular dichroism spectroscopy and by the analysis of UV-induced photoproducts. As the pH was lowered, poly[d(C-T)] underwent a conformational transition that was characterized by changes in the long-wavelength region (280–320 nm) of the CD spectrum. These changes have previously been interpreted as evidence for the formation of a core of stacked, protonated C·C$^+$ base pairs in a double-helical complex of poly[d(C-T)], with the thymidyl residues being looped out into the solvent [Gray, D. M., Vaughan, M., Ratliff, R. L., & Hayes, F. N. (1980) *Nucleic Acids Res. 8*, 3695-3707]. In the present work, poly[d(C-T)] was labeled with [U-^{14}C]cytosine and [*methyl*-^{3}H]thymine and irradiated at pH values both above and below the conformational transition point (monitored by CD spectroscopy). The distribution of radioactivity in uracil<>uracil dimers, uracil<>thymine dimers (the deamination products of cytosine<>cytosine and cytosine<>thymine dimers, respectively), and thymine-<>thymine dimers was then determined. As the pH was decreased, we found an increase in the yield of uracil<>uracil dimers and a decrease in the yield of uracil<>thymine dimers, which occurred concomitantly with the change in the CD spectrum. These changes were interpreted as evidence that a stacked C·C$^+$ base-paired structure was indeed formed by alternate cytosine bases in poly[d(C-T)] at acid pH, with the thymidyl residues being individually looped out of the structure. The dose-dependent kinetics of photoproduct formation and a loss of the long-wavelength CD band of the irradiated poly[d(C-T)] self-complex indicated that the structure was destabilized during irradiation, due to dimerization of the looped-out thymines. Taken together with the previous study showing that other cytosine-containing sequences may exist in a similar conformation (Gray et al., 1980), these results show that a new range of conformations is accessible to DNA.

Synthetic DNA polymers of various sequences have been shown to undergo acid-induced structural rearrangements as indicated by changes in their CD[1] spectra (Gray et al., 1978, 1980; Thiele et al., 1978a,b; Marck et al., 1978). Upon addition of acid to solutions of synthetic cytosine-containing DNA polymers, increased positive CD values are observed in the long-wavelength region (280–320 nm) of the spectrum, where the absorption is dominated by cytosine. For poly[d-

† This work was performed by D.M.B. in partial fulfillment of the requirements for the Ph.D. degree in the Molecular Biology Program, The University of Texas at Dallas. We are grateful for support by NIH Research Grant GM 19060 from the National Institute of General Medical Sciences and by Grant AT-503 from the Robert A. Welch Foundation. Work at the Los Alamos National Laboratory was supported by the U.S. Department of Energy. A preliminary report of this work was presented at the 27th Annual Biophysical Society Meeting (Brown et al., 1983).
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[1] Abbreviations: CD, circular dichroism; C<>C, C<>T, T<>T, U<>U, and U<>T, pyrimidine dimers (cyclobutyl dipyrimidines) of cytosine–cytosine, cytosine–thymine, thymine–thymine, uracil–uracil, and uracil–thymine, respectively; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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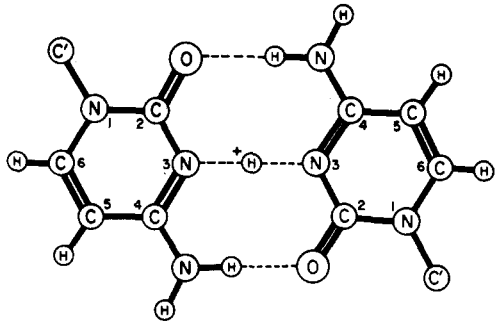

FIGURE 1: Hydrogen bonding between two cytosine residues in a C·C⁺ base pair, as proposed by Langridge & Rich (1963) for the acid self-complex of poly[r(C)]. Later work by Arnott et al. (1976) showed that the fiber diffraction pattern obtained by Langridge & Rich (1963) was from a stacked, single-stranded form of poly[r(C)]. However, base pairing of this type does exist in the crystal structure of cytosine-5-acetic acid (Marsh et al., 1962) and between the cytosines of a parallel dimer of CpA cocrystallized with proflavin (Westhof & Sundaralingham, 1980).

(C)], this type of CD change has been taken as evidence for the formation of stacked, protonated cytosine base pairs (C·C⁺ base pairs) (Gray & Bollum, 1974; Marck et al., 1978; Thiele et al. 1978a), which could be of the type proposed by Langridge & Rich (1963) and shown in Figure 1. For poly[d-(C-T)], a DNA polymer containing alternating cytosine and thymine bases, there is a marked similarity between the long-wavelength CD spectrum of this polymer under acidic conditions and that of the self-complex of poly[d(C)], which led to the proposal that there is a core of stacked C·C⁺ base pairs in the acid poly[d(C-T)] self-complex and that the thymidyl residues are looped out of the helix (Gray et al., 1980). Looped-out residues previously have been found for RNA copolymer–homopolymer duplexes containing mismatched bases (Lomant & Fresco, 1975), and noncomplementary residues are extrahelical in the duplexes formed by poly[d(G-G-A)] + poly[d(C-T)] (Evans & Morgan, 1983) and by the oligomers $d[CA_3CA_3G]$ + $d[CT_6G]$ (Morden et al., 1983). However, in these cases the noncomplementary residues are looped out of a core helix stabilized by Watson-Crick base pairs. The work by Gray et al. (1980) provided the first indication that self-complexes containing unusual base pairs might be stable even when every other base is looped out of both strands.

We have exploited the known photochemical properties of thymine and cytosine in polynucleotides in order to study the acid structure of poly[d(C-T)] and to correlate structural and CD spectral changes of the polymer as a function of pH. If poly[d(C-T)] under acid conditions does form a loop-out structure containing a core of stacked cytosines, then UV irradiation would be expected to result in an increase in the amount of C<>C formed and a concomitant decrease in the amount of C<>T formed, for the acid self-complex of the polymer relative to the single-stranded form of the polymer.

Materials and Methods

Synthesis and Isolation of Polydeoxynucleotides. Poly[d-(A-G)·d(C-T)] was synthesized as previously described (Gray & Ratliff, 1975) with [*methyl*-^{3}H]TTP (22 Ci/mmol, ICN Pharmaceuticals), [U-^{14}C]dCTP (48 mCi/mmol, Amersham), and [2-^{3}H]dATP (ICN Pharmaceuticals) as labeled substrates. The poly[d(C-T)] strand was isolated by preferential formic acid hydrolysis of the polypurine strand in poly[d(A-G)·d(C-T)] according to the method of Harwood & Wells (1970), followed by alkaline hydrolysis of the apurinic acid in 1 N NaOH and exhaustive dialysis against 0.05 M Na^+ (phosphate buffer), pH 7.9. The purified poly[d(C-T)] had a specific activity of 4.2 Ci/mol [U-^{14}C]cytidine (with label in both the base and sugar) and 40 Ci/mol [*methyl*-^{3}H]thymine. From the hydrolysis of a parallel sample of poly[d(C-T)] in which the polypurine strand was labeled with ^{14}C and the polypyrimidine strand was labeled with ^{3}H, we determined that there was less than 0.1% contamination of the isolated poly-[d(C-T)] strand by the homologous polypurine strand under our hydrolysis conditions. A nearest-neighbor frequency analysis of other batches of poly[d(A-G)·d(C-T)] synthesized by the same method showed that at least 98% of the polypyrimidine strand consisted of CpT and TpC nearest neighbors. The purified poly[d(C-T)] had a chain length of 100–560 nucleotides (weight-average length of 250), determined by the chromatographic method of Hayes & Mitchel (1969).

Poly[d(C)] labeled with [U-^{14}C]dCTP was the product of a terminal transferase synthesis performed by the procedure of Bollum (1966), except that 1 mM $CoCl_2$ was used instead of $MgCl_2$. The ratio of nucleotide to initiator was 1000:1, and 67% of the nucleotides was incorporated; thus, the average chain length of the synthesized poly[d(C)] should have been at least 670 nucleotides (Hoard et al., 1969). The reaction mixture was extracted 3 times with phenol and then twice with diethyl ether and was dialyzed exahustively against 0.05 M Na^+ (phosphate buffer), pH 7.9. The purified polymer had a specific activity of 19 mCi/mol [U-^{14}C]cytidine.

A sample of poly[d(C-G)] was synthesized by the procedure of Grant et al. (1972) and was dialyzed into 0.05 M Na^+ (phosphate buffer), pH 7.9.

Preparation of Standards. Radioactive U<>U, T<>T, and U<>T standards were prepared by using a germicidal lamp to irradiate frozen aqueous solutions of [5-^{3}H]uracil (22 Ci/mmol), [*methyl*-^{3}H]thymine (22 Ci/mmol), and a mixture of the two. [2-^{14}C]Cytosine (28 mCi/mmol) was used without any modification. These three labeled pyrimidines were purchased from Schwarz-Mann.

Sample Irradiation. Polymer samples (typically 0.5 mL) containing a least 10^6 dpm ^{14}C were irradiated at room temperature with constant stirring in a 1-cm path-length quartz cuvette with monochromatic light (280 ± 8 nm) at a fluence rate of 16.6 Wm^{-2}, according to previously described methods (Patrick & Gray, 1976). The irradiated polymers were all in 0.05 M Na^+ (phosphate buffer), pH 7.9.

Spectral Measurements and pH Changes. Absorption spectra were taken with a Cary-Varian Model 118 spectrophotometer, and CD spectra were obtained with a Cary Model 61 circular dichrometer, calibrated as previously described (Gray et al., 1980). CD values are given as $\epsilon_L - \epsilon_R$ in units of L·(mol of nucleotide subunit)$^{-1}$·cm^{-1} or as the absorbance difference $A_L - A_R$. Concentrations were determined from absorbance measurements and the extinction coefficients at 260 nm of 7500 L·mol^{-1}·cm^{-1} for single-stranded poly[d(C-T)] (Lee et al., 1979) and 5300 L·mol^{-1}·cm^{-1} for the self-complexed form of poly[d(C)] at neutral pH (Bollum, 1966). Samples were acidified by addition of dilute phosphoric acid; the total volume change caused by the addition of acid was always less than 1%. The pH values of samples were measured directly, using a Beckman Century SS1 pH water equipped with a Beckman 5-mm Futura combination electrode.

Isolation, Identification, and Quantitation of Photoproducts. Samples of irradiated polymers were immediately brought to neutral pH, if necessary, by the addition of NaOH and then were adjusted to 0.1 M Tris-HCl, pH 7.0, and placed in boiling water for 10 min. This procedure deaminates approximately 80% of the cytosine-containing dimers to their

corresponding stable uracil dimers (Varghese, 1971; Setlow et al., 1965). The samples were then dialyzed for at least 4 h against 0.01 M Tris-HCl, pH 7.0, at 4 °C. (Removal of phosphate ions was necessary since otherwise they inhibited the subsequent spermine precipitation step.) The dialyzed samples were transferred to lengths of Pyrex glass tubing sealed at one end. Approximately 100 μg of calf thymus DNA in 0.1 mL of water was added to each sample as a carrier, and the mixture was vortexed. Spermine tetrahydrochloride was added to give a final concentration of 5 mM, and the solutions were vortexed and placed at 4 °C overnight to precipitate the DNA (Hoopes & McClure, 1965). To collect the precipitated DNA, the glass tubes were placed on rubber cushions inside nitrocellulose tubes and centrifuged at 18000*g* in an SW25.3 rotor. This procedure routinely gave >96% recovery of the labeled DNA. Supernatants were removed, and about 0.5 mL of trifluoroacetic acid was added to each sample. The tubes were covered and placed in a 70 °C water bath for 30 min to dissolve the precipitated DNA. The other end of the glass tube was then sealed, and the samples were hydrolyzed for 90 min at 175 °C. When cool, the tubes were opened, and the trifluoroacetic acid was evaporated in a vacuum desiccator. The hydrolysates were taken up in 25 μL of deionized water and spotted onto preabsorbant zones on Whatman LK6DF silica thin-layer chromatography plates; each tube was rinsed twice with 10 μL of water, which was also spotted onto the preabsorbent zones. Routinely, more than 90% of the radioactivity was transferred from each tube to the plate. The preabsorbent zones were dried with a heat gun at room temperature.

The thin-layer chromatography plates were developed in 1-butanol–isobutyric acid–H_2O–28% ammonia (10:3.75:2.5:0.25) for 6 h. The 1-butanol contained 5% (w/v) 2,5-diphenyloxazole for fluorography, and the water was saturated with sodium tetraborate, pH 5.5. The distribution of radioactivity was visualized by fluorography on Kodak XOMAT-AR film at −70 °C. Standards, prepared as described above, were chromatographed on the same plate with the samples. The lanes for each sample and standard were fractionated into 2-mm zones and removed from the glass plate by application of Strip-Mix (Applied Sciences); the material from each zone was eluted by placing the 2-mm strip into a scintillation vial containing 0.5 mL of water. Toluene-based liquid scintillation fluid containing Triton X-100 was added, and radioactivity was measured in a Beckman LS7500 liquid scintillation counter. Measurements were corrected for quenching by the H-number method (Horrocks, 1977). The analysis was designed to detect acid-stable pyrimidine photoproducts amounting to 0.1% of the applied radioactivity.

Proton Uptake Measurements. Poly[d(C-T)] was dialyzed against 0.05 M Na^+ (phosphate buffer), pH 7.9. A 2-mL sample of the dialysis buffer was titrated at room temperature (24 °C) to pH 3.0 by addition of standardized 1.0 N HCl, with constant stirring and nitrogen gas bubbling through the solution. pH measurements were made as described above; the amount of HCl added was adjusted to give increments of 0.05–0.1 pH unit. The procedure was then repeated with a dialyzed sample of poly[d(C-T)] containing approximately 0.5 μmol of the polymer. Differences in the measured pH values were converted to hydrogen ion concentrations, and these were divided by the nucleotide concentration of poly[d(C-T)] to give the protons taken up per nucleotide.

Results and Discussion

Effect of pH on Spectral Properties of Poly[d(C-T)]. The CD spectra of poly[d(C-T)] at pH 7.9 and pH 5.0 (0.05 M Na^+) are shown in Figure 2. The spectrum of the polymer

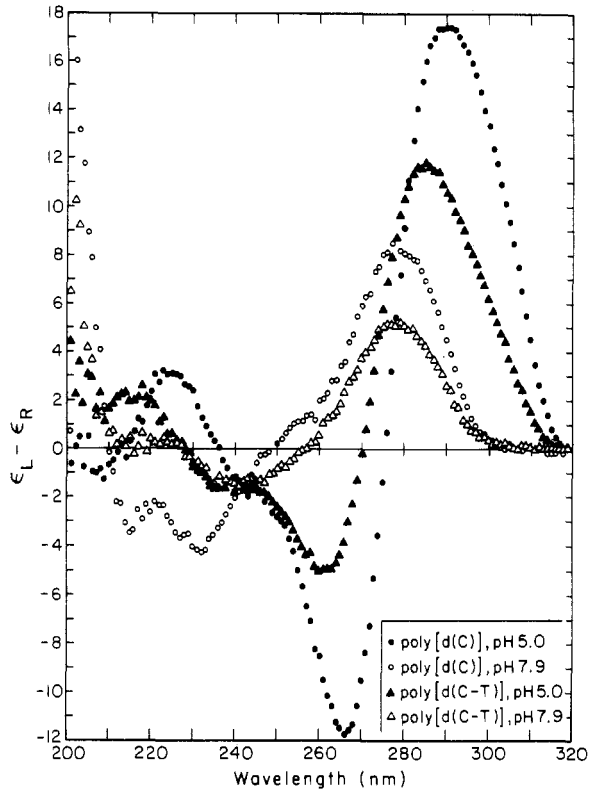

FIGURE 2: Circular dichroism spectra of poly[d(C)] and poly[d(C-T)] in a 0.05 M Na^+ at pH 7.9 and 5.0, 20 °C: poly[d(C)] at pH 5.0 (●) and at 7.9 (○); poly[d(C-T)] at pH 5.0 (▲) and at 7.9 (△).

at pH 7.9 was that of the single-stranded conformation and could be approximated with the CD contributions of TpC and CpT dinucleotides, as previously reported (Gray et al., 1980). At the lower pH, poly[d(C-T)] showed an enhanced positive CD band above 275 nm and a negative CD band at about 265 nm. These features have been interpreted as being the result of the formation of stacked, protonated $C{\cdot}C^+$ base pairs in an acid self-complex of the polymer (Gray et al., 1980). Comparison of the spectra of poly[d(C)] and poly[(C-T)] at acid and alkaline pH values showed that the acid-induced changes in the CD spectra were quite similar for the two polymers, particularly at wavelengths above 240 nm. The spectrum of the poly[d(C-T)] acid self-complex above 240 nm had about half the molar magnitude of the spectrum of the poly[d(C)] acid self-complex because CD values were expressed per mole of nucleotide and only half of the nucleotides in poly[d(C-T)], compared with all of the nucleotides in poly[d(C)], were capable of forming stacked $C{\cdot}C^+$ base pairs.

The poly[d(C-T)] acid self-complex, monitored by the CD at 283 nm, was formed cooperatively with a pK_a of 6.2 in 0.05 M Na^+, at 20 °C, as shown in Figure 3 (top panel). The self-complex was maximally stable in the region of pH 5–4 and was destabilized below pH 4. Coincident with the CD changes, 24% hypochromicity at 265 nm was observed as the pH was lowered to between 5 and 4. This value of hypochromicity upon forming an acid self-complex from single-stranded poly[d(C-T)] was consistent with the stacking of at least half of the bases in the poly[d(C-T)] acid self-complex, since 33% hypochromicity is observed at 265 nm upon forming an acid self-complex from single-stranded poly[d(C)] (Inman, 1964).

As seen in Figure 3 (bottom panel), poly[d(C-T)] readily took up protons between pH 6.5 and 5.9. The pK_a for the cooperative phase of the curve was 6.3, which was in good agreement with the midpoint of the transition as monitored

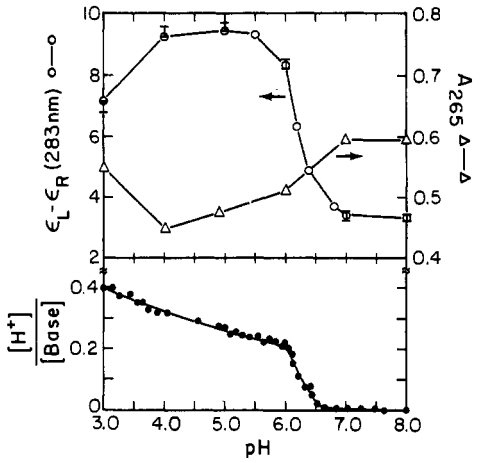

FIGURE 3: Changes in optical properties and proton uptake of poly[d(C-T)] in 0.05 M Na^+ as a function of pH. (Top panel) CD at 283 nm and absorbance at 265 nm. The error bars show the range of points from this and three additional experiments. (Bottom panel) Proton uptake, plotted as protons taken up per mole of base.

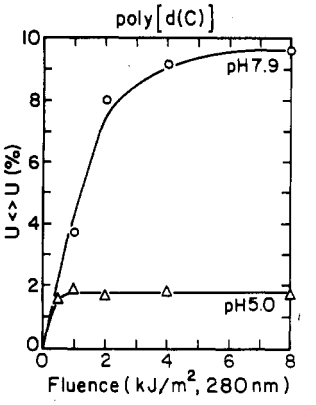

FIGURE 4: C<>C formation (measured as U<>U) as a function of fluence in poly[d(C)] irradiated at pH 7.9 (O) and 5.0 (Δ). The poly[d(C)] acid self-complex was stable at pH values ≤ 6.8 in 0.05 M Na^+ (Inman, 1964). It was irradiated at pH 5.0 in order to have results under the same solution conditions in which the poly[d(C-T)] self-complex was maximally stable. Polymer solutions irradiated to obtain the data for Figures 4–7 all contained 0.05 M Na^+ (phosphate buffer), pH 7.9.

by the CD at 283 nm (Figure 3, top panel). The number of protons per base was 0.22 at pH 5.9 and reached the predicted value of 0.25 proton per base (1 proton for each C·C+ base pair) at pH 5.1. The pK_a for the poly[d(C-T)] transition (6.3) was closer to the pK_a of deoxycytidine (4.3; Fox & Shugar, 1952) than is the pK_a of the poly[d(C)] transition (7.4; Inman, 1964). Thus, the pH had to be brought closer to the pK_a of dC before C·C+ base pair formation was favored in poly[d(C-T)] relative to poly[d(C)]. This was in agreement with differences in thermal stabilities of the polymers that show the poly[d(C-T)] acid self-complex to be less stable than that of poly[d(C)] at the same pH (Gray et al., 1980).

Poly[d(C-T)] continued to take up protons as the pH was reduced below 5.1 (Figure 3, bottom panel). Below pH 4 there was a loss of CD at 283 nm and of the hypochromicity at 265 nm (Figure 3, top panel). Protonation of the poly[d(C-T)] self-complex to an extent greater than 0.5 proton per cytosine thus eventually destabilized the C·C+ base pairs, an effect also seen for poly[d(C)] and poly[r(C)] acid self-complexes (Inman, 1964; Hartman & Rich, 1965). In summary, the proton uptake and spectral properties of poly[d(C-T)] at acid pH were characteristic of stacked C·C+ base pairs, as in poly[d(C)], and implied that the thymidyl residues were extrahelical in the acid form of poly[d(C-T)].

pH Dependence of C<>C Formation in Poly[d(C)]. Our expectation that C<>C formation would be a sensitive probe of the proposed poly[d(C-T)] loop-out conformation in acid conditions was based on the assumption that the cytosine residues in a double helix formed by stacked C·C+ base pairs would indeed dimerize when irradiated with 280-nm light. The poly[d(C)] acid self-complex was taken to be the model of a structure containing stacked C·C+ base pairs. The fluence dependence of cytosine dimerization in poly[d(C)] in the single-stranded (pH 7.9) and self-complexed (pH 5.0) forms is shown in Figure 4. Cytosine dimerization (measured as U<>U formation) in single-stranded poly[d(C)] at pH 7.9 reached a photosteady state at a fluence of 8.0 kJ·m^{-2}, where about 9.5% of the cytosine radioactivity was recovered as U<>U. This value was comparable to the value of 11% U<>U observed from irradiation of the poly[d(I)-d(C)] duplex (Setlow et al., 1965). Irradiation of the poly[d(C)] self-complex at pH 5.0 resulted in a photosteady state of cytosine dimerization at 1 kJ·m^{-2}, with a yield of U<>U of about 1.8%, 5-fold lower than at pH 7.9. The shared proton in a C·C+ base pair might have been responsible for this reduction in the photosteady state yield of C<>C. This explanation is consistent with a previous observation that the yield of C<>C decreases 5-fold in irradiated CpC dinucleotide as the pH is decreased from 7 to 3 (Freeman et al., 1965). Alternatively, the reduction in C<>C yield in the acid self-complex of poly[d(C)] might have been the result of spatial constraints imposed by the structure; that is, stacked C·C+ base pairs might not have had sufficient flexibility to allow maximum dimerization of the cytosines. Whatever the explanation for the difference in yield, the fact that C<>C dimers were formed in poly[d(C)] under both acid and alkaline conditions made feasible the analysis of C<>C dimer formation in poly[d(C-T)] under similar solution conditions and provided a measure of the yield of C<>C we could expect from the dimerization of stacked cytosines in a loop-out structure of poly[d(C-T)].

pH Dependence of Photoproduct Formation in Poly[d(C-T)]. The yields of photoproducts in poly[d(C-T)] and the CD spectrum of the polymer in 0.05 M Na^+ (phosphate buffer) were measured at various pH values as the pH was decreased by the addition of dilute phosphoric acid. Aliquots were removed from the sample at different pH values, irradiated, and then analyzed for photoproduct formation as described under Materials and Methods. The chromatographically separated radioactive pyrimidines and their respective photoproducts were visualized on X-ray film by fluorography, shown in Figure 5, before elution and quantitation. An unirradiated poly[d(C-T)] control sample at pH 5 had thymine, uracil, and cytosine bands, but no bands with the relative mobilities of the various Pyr<>Pyr photoproducts. The presence of uracil was due to cytosine deamination during trifluoroacetic acid hydrolysis (Varghese, 1971). It is apparent from the film that the amount of U<>U increased and the amount of U<>T decreased as the pH decreased from 8 to 5.

Quantitation of the yields of the various photoproducts at decreasing pH values for a constant fluence of 6 kJ·m^{-2} led to the values given in Table I. The data for U<>T and U<>U formation are also shown in Figure 6. The U<>U yield (not corrected for pH dependence) showed a 3-fold *increase* as the pH decreased from 8 to 4 (Table I), although the effect of reduced pH was to *decrease* the yield of U<>U in the case of irradiated poly[d(C)], as described above. When corrected for the effect of protonation on cytosine dimerization, the increase in fractional yield of cytosine-derived U<>U from poly[d(C-T)] with decreasing pH was nearly 8-fold (Table I

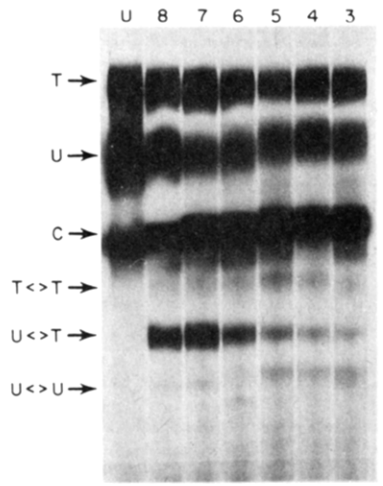

FIGURE 5: Fluorogram showing the distribution of radioactivity in hydrolysis products from poly[d(C-T)] irradiated with 6.0 kJ·m^{-2} of 280-nm light at various pH values: (lane U) unirradiated; (lanes 8–3) poly[d(C-T)] samples irradiated at pH values of 8–3. The various radioactive bands were identified by comparison with the relative mobilities of standards. The origin is near the bottom of the figure. The U<>U band at pH 6 had a slightly lower mobility than was observed in the other samples. Such a decreased mobility for U<>U occurred at random. Although the reason for this effect was not understood, it did not influence our conclusions.

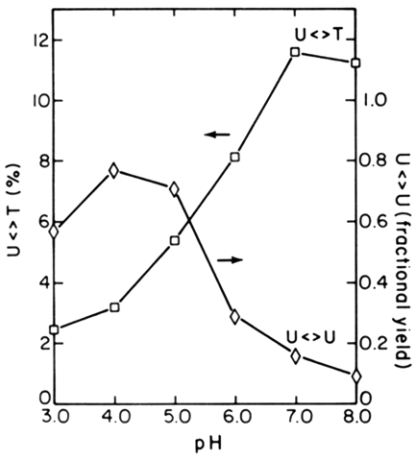

FIGURE 6: C<>T (□) and C<>C (◇) photoproduct formation (measured as U<>T and U<>U, respectively) in poly[d(C-T)] as a function of pH, from the same chromatographed samples used for the fluorogram in Figure 5. Values plotted are from columns 2 and 5 of Table I.

and Figure 6). The yield of U<>U from poly[d(C-T)] was about 70% of that obtained from poly[d(C)] under comparable conditions. It is evident from Figure 6 that the yield of U<>U from poly[d(C-T)] was similar in its pH dependence to the changes in the CD and absorption data (Figure 3), the U<>U yield being maximal at pH values of 4–5. The yield of U<>U from poly[d(C-T)] dropped when the pH was decreased below 4, also in agreement with the spectral data. (The fact that the C<>C formed in poly[d(C-T)] at acid pH reached over 70% of the C<>C formed in poly[d(C)] eliminated the possibility that C<>C might only be formed in 1–2% of the poly[d(C-T)] polymer that did not have a strictly repeating sequence; see Materials and Methods.)

The yield of U<>T from poly[d(C-T)] showed a pH dependence opposite to that observed for U<>U (Table I and Figure 6). U<>T, which was expected to be the predominant dimer obtained from irradiation of single-stranded poly[d(C-T)], decreased nearly 5-fold as the pH decreased from 8 to 3. This was consistent with the formation of a conformation

Table I: Photoproducts Obtained from Poly[d(C-T)] Irradiated with 6.0 kJ·m^{-2} of 280-nm Light, as a Function of pH

	photoproduct			
pH (±0.1)	U<>T (%)[a]	T<>T (%)[a]	U<>U (%)[a]	U<>U (fractional yield)[b]
8	11.18 ± 0.09	2.32 ± 0.15	0.38 ± 0.16	0.09 ± 0.06
7	11.15 ± 0.12	2.70 ± 0.08	0.55 ± 0.10	0.16 ± 0.08
6	8.10 ± 0.12	3.10 ± 0.13	0.75 ± 0.11	0.30 ± 0.12
5	5.35 ± 0.14	3.90 ± 0.09	1.29 ± 0.09	0.71 ± 0.14
4	3.18 ± 0.11	3.48 ± 0.10	1.40 ± 0.08	0.77 ± 0.09
3	2.40 ± 0.15	3.12 ± 0.12	1.05 ± 0.15	0.57 ± 0.16

[a] Pyr<>Pyr yields were calculated as the percentage of the total radioactivity in the parent pyrimidines. For U<>T, equivalent results were obtained by calculating the percentage of ^{3}H or ^{14}C in the dimer, as expected; the values shown were calculated from the percentage of ^{3}H in the dimer since the specific activity of ^{3}H was higher than that of ^{14}C. Errors give the range of values from two experiments. [b] The U<>U data were corrected for the influence of protonation on cytosine dimerization by expressing the U<>U yield from poly[d(C-T)] as a fraction of the yield from poly[d(C)] at the same pH. For example, the yield of U<>U in poly[d(C)] irradiated with 6.0 kJ·m^{-2} at pH 5 was 1.82%. The yield of U<>U from poly[d(C-T)] irradiated at pH 5 with the same fluence was 1.29%, or 0.71 of that from poly[d(C)] under the same conditions. For pH values other than 8 (≃7.9) and 5, the yield of U<>U from poly[d(C)] was taken to be the value from a straight-line fit to the data obtained at pH 7.9 and 5 (from Figure 4).

in which cytosine and thymine residues were no longer spatially adjacent to each other under acid conditions. In fact, the actual yield of U<>T that would be formed at a fluence of 6 kJ·m^{-2} in a stable acid self-complex of the polymer probably would be less than shown by these data, since a significant fraction of the U<>T obtained at this fluence resulted from irradiation of a disrupted poly[d(C-T)] structure, as discussed in the following section. Some fraction of the observed decrease could have been due to cytosine protonation, but there was no good way to estimate the degree of such an effect independent of the effect of a change in structure.

Although not as dramatic, the yield of T<>T from poly[d(C-T)] as a function of pH resembled that of U<>U. The yield of T<>T increased from 2.3% of the total thymine at pH 8 to 3.9% at pH 5 (Table I). The significance of T<>T formation will be discussed in the next section.

Fluence Dependence of Photoproduct Formation in Poly[d(C-T)] Irradiated at pH 5. Samples of poly[d(C-T)] at pH 5 were irradiated with fluences of up to 8.0 kJ·m^{-2} of light at 280 nm and then were analyzed for photoproducts following the procedure described under Materials and Methods. The data from two such experiments are plotted in Figure 7 (bottom panel) along with concomitant dose-dependent changes in the long-wavelength CD band of poly[d(C-T)] (top panel). From the bottom panel of Figure 7, it appears that C<>C was formed even at relatively low fluences, where very little C<>T or T<>T was detected; only at fluences > 2.0 kJ·m^{-2} were C<>T and T<>T formed in proportionately greater amounts.

A reason for the dissimilar fluence dependence of formation of the different Pyr<>Pyr in the poly[d(C-T)] self-complex became apparent upon examinating the conformational integrity of acid self-complexes of different cytosine-containing polymers upon exposure to UV light. The long-wavelength positive CD bands of the acid self-complexes of poly[d(C-T)], poly[d(C)], and poly[d(C-G)] were monitored as a function of fluence of 280-nm light. The CD spectra of all three of these polymers undergo an acid-dependent transition characteristic of the formation of stacked C·C^{+} base pairs (Figure 2; Thiele et al., 1978b). It has been previously suggested that poly[d(C-G)] forms a loop-out structure (at pH 2.5) like that

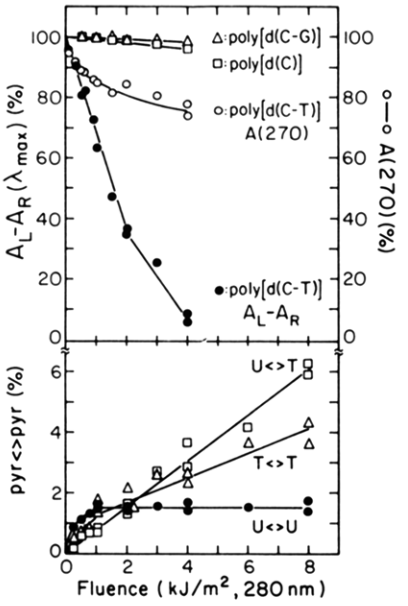

FIGURE 7: Changes in optical properties of poly[d(C)], poly[d(C-T)], and poly[d(C-G)] and photoproduct formation in poly[d(C-T)] at pH 5.0, as a function of fluence. (Top panel) CD at the wavelength peak of the positive long-wavelength CD band for poly[d(C)] at pH 6.8 (□), for poly[d(C-T)] at pH 5.0 (●), and for poly[d(C-G)] at pH 2.5 (Δ), as a function of fluence of 280-nm light. The optical density at 270 nm of poly[d(C-T)] irradiated at pH 5.0 (○) is also plotted as a function of fluence. (Bottom panel) Formation of C<>T (□), T<>T (Δ), and C<>C (●) (measured as U<>T, T<>T, and U<>U, respectively) as a function of fluence in poly[d(C-T)] irradiated at pH 5.0. Data plotted were from two experiments.

of poly[d(C-T)], but with looped-out guanyl residues (Gray et al., 1980). As seen in the top panel of Figure 7, only the poly[d(C-T)] acid self-complex had a secondary structure that was sensitive to irradiation. About two-thirds of the long-wavelength CD of the poly[d(C-T)] acid self-complex was lost at a fluence of 2 kJ·m^{-2}. Since the absorbance of poly[d(C-T)] decreased by only 20% at this fluence (Figure 7, top panel), the loss of the CD at 283 nm was not simply due to photochemical elimination of the chromophore. The fact that the long-wavelength CD of the poly[d(C)] self-complex was not significantly affected at these fluences argued against the dimerization of the stacked C·C$^+$ base pairs being responsible for the loss of structure in poly[d(C-T)]. The observation that the long-wavelength CD of the poly[d(C-G)] acid self-complex was unaffected at these fluences suggested that the loss of structure in poly[d(C-T)] was not due to any inherent instability of the loop-out conformation in polydeoxynucleotides of alternating sequence containing a core of irradiated C·C$^+$ base pairs. (Purines are presumably photochemically unreactive at these fluences; Elad, 1976). In sum, therefore, these data implied that it was the photochemical reactivity of the thymine residues in the poly[d(C-T)] acid self-complex that was responsible for the irradiation-induced collapse of its structure.

The fluence dependence of photoproduct formation in the poly[d(C-T)] acid self-complex (Figure 7, bottom panel) was understandable in light of the above spectral data. C<>C was the predominant photoproduct obtained at fluences of ≤1 kJ·m^{-2}, although the irradiation-induced loss of structure measured by CD proceeded at a high rate up to 2 kJ·m^{-2}. It was at this latter fluence that the yields of C<>T and T<>T overtook that of C<>C. Thus, we concluded the following: (1) Cytosine dimerization in the poly[d(C-T)] self-complex reached a photosteady state yield of about 1.4% at relatively low fluences where the structure (monitored by CD) was still about 75% intact. (Cytosine dimerization in poly[d(C)] at acid pH values also reached a photosteady state at low fluences, ≤1 kJ·m^{-2}, as shown in Figure 4.) It seemed likely from the CD data on other sequences that cytosine dimerization had little direct effect on the stability of the structure of the poly[d(C-T)] self-complex. (2) It was the presence of the thymidyl residues that resulted in the destabilization of the poly[d(C-T)] structure, the structure of the acid self-complex apparently being flexible enough that the thymines could occasionally come close enough to dimerize. According to our model of the poly[d(C-T)] acid self-complex (shown in Figure 8 and discussed below), looped-out thymines that dimerize would be in opposite strands and could destabilize the structure to the point where C·C$^+$ base pairs would be disrupted with a loss of CD at 283 nm. (3) Once the C·C$^+$ base pairs were disrupted, a collapsed form of the polymer resulted that contained all possible pyrimidine stacks and in which C<>T and T<>T could continue to accumulate since their photosteady

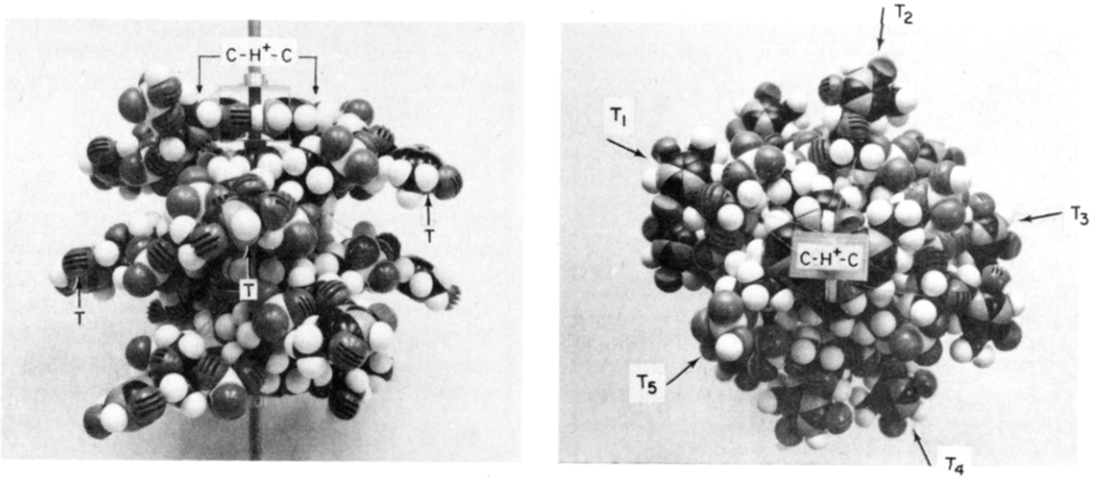

FIGURE 8: Space-filling model of a possible double-helical structure of the poly[d(C-T)] self-complex, consisting of a core of C·C$^+$ base pairs and looped-out thymidyl residues: (a) side view, showing a C·C$^+$ base pair (top) and three of the looped-out thymine bases from one of the strands (middle); (b) top view, showing a C·C$^+$ base pair (center) and looped-out thymine bases (T_1–T_5, in decending order) from one of the strands.

states had not yet been reached. C<>C did not continue to form in the collapsed poly[d(C-T)] since the photosteady state for cytosine dimerization had been reached at a fluence of 1 kJ·m^{-2}, at which the structure was still about 75% intact. (This latter fact ruled out the possibility that C<>C might be produced only in the disrupted state and might not be diagnostic of stacked C·C$^+$ base pairs. If C<>C were produced only in the disrupted state, the yield of C<>C should have continued to rise until fluences of at least 4 kJ·m^{-2} were reached, at which the polymer was completely disrupted; see Figure 7.)

Model. A plausible loop-out model of the poly[d(C-T)] acid self-complex is shown in Figure 8. The model shows a core of stacked C·C$^+$ base pairs, of the type shown in Figure 1, oriented perpendicular to the axis of a double helix from which thymidyl residues are looped out. The looped-out thymines give the structure a radius of approximately 16 Å. The strands are parallel with about four C·C$^+$ base pairs per helical turn (i.e., four C-T dinucleotide repeats). The nucleotides in this model all have anti sugar–base conformations and C2′-exo sugars. Angles of the C3′–O3′, O3′–P, P–O5′, O5′–C5′, and C5′–C4′ bonds are *t*, *t*, g^-, *t*, and g^-, respectively, along the backbone from one extrahelical to the next intrahelical residue. The same bonds from one intrahelical to the next extrahelical residue are *t*, *t*, g^+, *t*, and g^+, respectively. It is possible that a poly[d(C-T)] loop-out structure could also be built having an alternative base pairing and antiparallel strands, as proposed for C·C$^+$ base pairs in the self-complex of the oligomer d-($C_4A_4T_4C_4$) (Gray et al., 1984). However, we have not explored this or other possible models of a loop-out structure for poly[d(C-T)].

The closest thymines are those connected to opposite strands across a helix groove and are on average about 8 Å apart. The occasional dimerization of such pairs of thymines would force the thymines involved to remain closely stacked. Thymine dimerization could coincide with the disruption of neighboring C·C$^+$ base pairs and the local collapse of the loop-out structure into a more random structure, although held together by the T<>T dimers that are initially formed. It probably was in such a collapsed structure that most of the C<>T and T<>T dimers were formed, as indicated by the kinetic data presented above.

Conclusions

The pH dependences of the photoproduct yields in poly[d-(C-T)] upon irradiation with 280-nm light were consistent with the hypothesis based on CD measurements (Gray et al., 1980) that, under acid conditions, poly[d(C-T)] forms a loop-out structure consisting of a core of C·C$^+$ base pairs and individually looped-out thymidyl residues. The essential result of the photochemical studies was that, in the pH range where CD spectra suggest the formation of a poly[d(C-T)] self-complex with stacked C·C$^+$ base pairs, C<>C formation became the favored photochemical event in poly[d(C-T)] irradiated with low fluences.

We conclude that poly[d(C-T)] forms a double-helical loop-out structure at acid pH, with the general features shown in the model in Figure 8. The pK_a for C·C$^+$ base-pair formation in long stretches of C's can be significantly greater than the pK_a of 6.2–6.3 for their formation in poly[d(C-T)], so that C·C$^+$ base pairs are stable in poly[d(C)] at neutral pH (Inman, 1964). Also, it has now been shown that C·C$^+$ base pairs can form between antiparallel strands of DNA (Gray et al., 1984). Thus, stretches of polypurine–polypyrimidine sequences in natural DNAs might form loop-out structures with a significant probability, even at physiological pH, by back-folding of the polypyrimidine strand. Natural DNA sequences that have this potential include the *Drosophila virilis* DNA sequence recently found that contains the repeating theme (C-T)$_{8-18}$(C)$_{4-5}$ for about 400 bases (Tautz & Renz, 1984) and the chicken repetitive DNA sequence that consists of about 32 tandem repeats of C-C-T-C-T in one strand (Dybvig et al., 1983). The latter sequence has been reported to undergo a pH-dependent structural change that makes it sensitive to the single-strand specific endonuclease from *Neurospora crassa* at a pH of 4.5 or 4.8 but not at pH values of 5.5 or above, although pH 8 is optimal for the *N. crassa* enzyme (Dybvig et al., 1983). Another sequence that could form a loop-out structure is the sequence T-C-C-C-T-C-C-C-T-T-C-C-T-C-C-C-T-C-C-C-T, which flanks the 5′-end of the chicken pro-α2(I) collagen gene and in which an endonuclease S1 sensitive site is induced by supercoiling of recombinant plasmids containing the sequence (Finer et al., 1984). Such sequences do not have low melting temperatures, are not palindromic, and cannot form the left-handed Z conformation to give B to Z junctions. Whether loop-out structures stabilized by C·C$^+$ base pairs are involved in the structural changes or in vivo functions of such sequences remains to be investigated. The possibility of such structures should be kept in mind, however, when considering structures and functions of noncoding DNA sequences.

Registry No. Poly[d(C-T)], 49717-92-2; cytosine, 71-30-7; thymine–thymine dimer, 28806-14-6; uracil–uracil dimer, 28806-15-7.

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N-Terminal Analogues of Cecropin A: Synthesis, Antibacterial Activity, and Conformational Properties†

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Received August 15, 1984

ABSTRACT: Six analogues of the 37-residue antibacterial peptide cecropin A were synthesized by the solid-phase method: cecropin A-(2–37), [Glu^2]cecropin A, [Pro^4]cecropin A, [Glu^6]cecropin A, [Leu^6]cecropin A, and [Pro^8]cecropin A. Their antibacterial activities against four test organisms were determined and related to conformational changes observed in their CD spectra and were discussed on the basis of a previously proposed amphipathic α-helix model. An aromatic residue in position 2 was shown to be important for activity against all tested bacteria. The highly α-helical 1–11 region of cecropin A did not appear to play a significant role in its activity against *Escherichia coli* but was clearly involved in its interaction against *Pseudomonas aeruginosa*, *Bacillus megaterium*, and *Micrococcus luteus*.

The induction of immunity in the pupae of the North American silk moth *Hyalophora cecropia* elicits a powerful antibacterial response characterized by the appearance of the cecropins, and of several other immune proteins in the insect hemolymph (Boman & Steiner, 1981). Cecropin A, a major contributor to this induced immunity, was recently synthesized by us (Andreu et al., 1983). Its antibacterial activity had been previously discussed in terms of a model consisting of two α-helical segments extending from the N-terminus to residue Ala^{22} and from residue Ala^{25} to the C-terminus with a major disruption of the helix caused by residues Gly^{23} and Pro^{24} (Merrifield et al., 1982). This model could be adjusted to offer a distribution of charged and hydrophobic residues that closely conformed to an ideal amphipathic helix (Segrest et al., 1974; Assman & Brewer, 1974; Kaiser & Kezdy, 1984) (Figure 1). We had also previously shown that both N- and C-terminal regions are needed to confer specificity of action to cecropin A. In particular, a considerable loss of activity against most of the selected test organisms was detected when the two N-terminal residues of cecropin A (Lys^1, Trp^2) were removed (Andreu et al., 1983).

In order to test the proposed model and thus achieve a better understanding of the mechanism of action of cecropin A, we have prepared six analogues having single-residue modifications: cecropin A-(2–37), [Glu^2]cecropin A, [Pro^4]cecropin A, [Glu^6]cecropin A, [Leu^6]cecropin A, and [Pro^8]cecropin A. The N-terminal region has been selected both for its synthetic convenience and for its already established role in the specificity of the molecule. The six synthetic analogues have been tested against two Gram-negative and two Gram-positive organisms, and their antibacterial activity is discussed and related to the conformational changes introduced in the molecules.

Experimental Procedures

Materials. Benzhydrylamine hydrochloride resin (0.56 mmol of N/g) was purchased from Beckman, Palo Alto, CA.

† This research was supported by Grant AM 01260 from the U.S. Public Health Service (to R.B.M.) and by Grant B2453 from the Swedish Natural Science Research Council (to H.G.B.). D.A. was supported by a postdoctoral fellowship from the Fundación Juan March, Spain, and by a grant from KabiGen AB, Sweden.

0006-2960/85/0424-1683$01.50/0