Structure, Dynamics, and Energetics of Deoxyguanosine·Thymidine Wobble Base Pair Formation in the Self-Complementary d(CGTGAATTCGCG) Duplex in Solution†

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ABSTRACT: Nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) have been used to investigate the helix-to-coil transition of the duplex formed by the self-complementary dodecamer $d(C_1G_2T_3G_4A_5A_6T_6T_5C_4G_3C_2G_1)$ (henceforth called 12-mer GT duplex) containing dG·dT interactions at position 3. These results are compared with the corresponding d(CGCGAATTCGCG) dodecamer duplex (henceforth called 12-mer GC duplex) containing standard Watson–Crick dG·dC base pairs at position 3 [Patel, D. J., Kozlowski, S. A., Marky, L. A., Broka, C., Rice, J. A., Itakura, K., & Breslauer, K. J. (1981) *Biochemistry* (first paper of four in this issue)]. The dG·dT interaction in the 12-mer GT duplex was monitored at the imino protons of both bases in the pair, the nonexchangeable protons of the thymidine residue, and the phosphate resonances at the modification site. We demonstrate dG·dT wobble base pair formation involving two imino proton–carbonyl group hydrogen bonds, based on a nuclear Overhauser effect between the adjacent interstrand guanosine and thymidine imino protons in the pair. Furthermore, the base pairing on either side of the dG·dT wobble base pair remains intact. The wobble base pair formation results in a small decrease in stacking of the thymidine residue with adjacent base pairs and a possible conformational change at two phosphodiester linkages. The nonexchangeable base proton markers at the dG·dT wobble pair at position 3 and the nonterminal Watson–Crick base pairs at positions 2, 4, 5, and 6 of the 12-mer GT duplex exhibit a common transition midpoint of ~52 °C in 0.1 M phosphate solution. Differential scanning calorimetry also demonstrates that replacing two dG·dC base pairs with two dG·dT base pairs reduces the melting temperature of the dodecanucleotide duplex by ~20 °C. Furthermore, we measure a calorimetric enthalpy change of 106 kcal (mol of double strand)$^{-1}$ for the 12-mer GT duplex in 0.1 M NaCl. This enthalpy change is of similar magnitude to the corresponding value measured for the 12-mer GC duplex. These calorimetric results are consistent with the dG·dT wobble pair stacking with adjacent pairs in the 12-mer GT duplex to about the same extent as a regular dG·dC base pair. The model-dependent van't Hoff enthalpies are found to be much smaller than the calorimetrically measured values, thereby indicating that the transition from duplex to single strand involves intermediate states. The size of the cooperative melting unit remains unchanged at 9 ± 1 base pairs despite the wobble base pair induced destabilization of the 12-mer GT duplex relative to the 12-mer GC duplex.

A great deal of our knowledge concerning the molecular forces that stabilize RNA structures has been obtained from systematic investigations of specially designed and synthesized ribooligonucleotides possessing the various structural features found in naturally occurring RNA molecules (Uhlenbeck et al., 1971, 1973; Craig et al., 1971; Martin et al., 1971; Porschke et al., 1973; Gralla & Crothers, 1973; Borer et al., 1974; Bloomfield et al., 1974; Breslauer et al., 1975).

Fewer corresponding investigations have been conducted on DNA structures due primarily to a lack of available deoxyoligomers (Burd et al., 1975; Selsing & Wells, 1979). Most early workers studied complementary homopolymers and self-complementary, alternating copolymers (Wells & Wartell, 1974; Arnott et al., 1975; Wells et al., 1977; Arnott, 1977; Leslie et al., 1980). Although important, the lack of sequence heterogeneity caused the information content of these molecules to be somewhat limited. Furthermore, in the absence of controlled sequence modification, it was difficult to correlate a given experimental observation with a specific molecular event. Thus, the effects of mismatches, bulges, and internal loops on the stability of DNA structures are just beginning to be understood (Dodgson & Wells, 1977; Early et al., 1977; Selsing et al., 1978; Wallace et al., 1979; Cornelis et al., 1979; Haasnoot et al., 1980).

The role of non-Watson–Crick base pairing in RNA and DNA duplexes has been investigated (Schimmel & Redfield, 1980; Cornelis et al., 1979; Wallace et al., 1979; Dodgson & Wells, 1977; Gillam et al., 1975; Topal & Fresco, 1976). A common mismatch involves noncomplementary purine·pyrimidine pairing such as G·U interactions observed in the stem regions of most transfer RNA sequences (Schimmel & Redfield, 1980) and dG·dT interactions observed in the poly(dG-dT) duplex (Early et al., 1978).

We have synthesized a series of deoxyribooligonucleotides that systematically differ by the replacement or addition of one or more nucleotides and have investigated the consequences of these controlled structural changes by nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC).

Nuclear magnetic resonance permits the resolution and assignment of proton markers on individual base pairs in DNA fragments (Patel & Canuel, 1976; Kallenbach et al., 1976; Early et al., 1977). Therefore, it is ideally suited for investigating the effects of specific chemical modifications in a DNA duplex by monitoring the hydrogen bonding, base pair overlaps and the phosphodiester backbone at a modification site.

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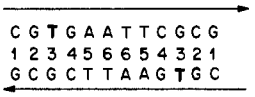
Chart I



Application of differential scanning calorimetry to the investigation of two duplex structures differing by a single structural alteration allows the energetic contribution associated with the specific modification to be defined. The additional insights derived from using both NMR and DSC to investigate helix–coil transitions of oligo- and polynucleotides have been demonstrated previously (Marky et al., 1981a,b).

This paper describes the NMR and calorimetric parameters for the self-complementary 12-mer GT duplex d-(CGTGAATTCGCG), which contains two non-Watson–Crick dG·dT interactions at position 3 from each end of the duplex (Chart I). These data are compared with related parameters for the self-complementary 12-mer GC duplex d-(CGCGAATTCGCG), which contains standard Watson–Crick pairing (Patel et al., 1981).

Experimental Procedures

Synthesis. The d(CGTGAATTCGCG) 12-mer GT was prepared by the modified triester method (Hirose et al., 1978) by coupling the protected trimer DM–CGT–PO$^-$ with the protected nonamer HO–GAATTCGCG–OBz (Patel et al., 1981), followed by deprotection and purification as described below.

The protected trimer was synthesized by coupling 0.6 mmol of DM–CG–PO$^-$ with 0.4 mmol of HO–T–PCE in the presence of 0.8 mmol of TPSTe condensing agent. The DM–CGT–PCE was isolated following workup and silica gel chromatography of the reaction product.

DM–CGT–PCE (0.135 g) was deblocked at its 3′ end and coupled with 0.22 g of HO–GAATTCGCG–OBz in the presence of TPSTe condensing agent. The protected 12-mer GT was chromatographed on silica gel, deblocked with acid and base, and rechromatographed on DE-23 ion-exchange column to yield 470 OD[1] unit of d(CGTGAATTCGCG) in pure form.

NMR Spectra. The NMR experiments were carried out on the spectrometers described in the preceding paper (Patel et al., 1981).

Calorimetry. The differential scanning calorimetry was carried out on a Microcal-1 instrument. The details of a typical calorimetric experiment are described under Experimental Procedures of the preceding paper (Patel et al., 1981). The data are obtained as excess heat capacity vs. temperature. The concentration of the 12-mer GT was calculated with an extinction coefficient of 1.13×10^5 M^{-1} in strands at 25 °C (Cantor & Warshaw, 1970).

Results

The 12-mer GT sequence exhibits 2-fold symmetry and contains dG·dC base pairs at positions 1, 2, and 4, dA·dT base pairs at positions 5 and 6, and a dG·dT interaction at position 3 in the self-complementary duplex (Chart I).

Nuclear Magnetic Resonance. (A) Imino Proton Line Widths. The 360-MHz proton Fourier transform NMR spectrum (9–15 ppm) of the 12-mer GT duplex in 0.1 M phosphate and H_2O, pH 5.8, at 1 °C is presented in Figure 1A. This spectral region contains guanosine H-1 and thymidine H-3 imino exchangeable protons that participate in

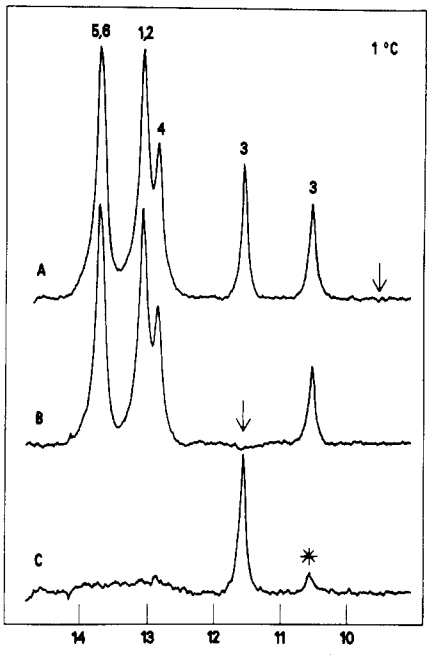


FIGURE 1: Nuclear Overhauser effect between imino protons of the dG·dT pair of the 12-mer GT duplex in 0.1 M phosphate, 1 mM EDTA, and 4:1 $H_2O/^2H_2O$, pH 5.8, at 1 °C. The decoupling pulse is applied at ~9.5 ppm in a resonance-free region in (A) and at the thymidine imino proton in the dG·dT pair at ~11.6 ppm in (B). The difference spectrum (C) shows a ~15% negative NOE at the guanosine imino proton in the dG·dT pair at ~10.6 ppm (designated by asterisk). The spectra were recorded by nulling the H_2O signal by using a Redfield 214 low-power pulse sequence. The decoupler was on for 100 ms, and the pulse repetition rate was 1 s. The signal to noise of the spectrum was improved by applying a 10-Hz line broadening contribution. A smoothing routine was applied to remove the curvature in the base line associated with the 214 pulse sequence.

intermolecular hydrogen bonds or are otherwise shielded from solvent (Kearns et al., 1971; Hilbers, 1979). It has been established for imino protons hydrogen bonded to ring nitrogens that the thymidine H-3 protons of dA·dT Watson–Crick base pairs resonate ~1 ppm downfield from the guanosine H-1 protons of dG·dC Watson–Crick base pairs in the spectral range 12.5–14.5 ppm (Hilbers, 1979). By contrast, imino protons either shielded from solvent or hydrogen bonded to carbonyl oxygens resonate to higher field between 10.0 and 12.0 ppm (Young & Kallenbach, 1978; Early et al., 1978; Cornelis et al., 1979). The two imino protons superimposable at 13.82 ppm are assigned to the two dA·dT base pairs, the imino protons at 13.26, 13.21, and 12.97 ppm are assigned to the three dG·dC base pairs, and imino protons at 11.78 and 10.58 ppm are assignable to the thymidine H-3 and guanosine H-1 in the dG·dT interaction in the 12-mer GT duplex at −5 °C (Table I).

We have monitored the imino proton spectral region in the 12-mer GT duplex in 0.1 M phosphate, pH 7.7, as a function of temperature. Typical spectra of the 12-mer GT duplex at 23, 35, and 47 °C are presented in Figure 2. The line width data for the guanosine H-1 imino protons at positions 1–4 in the 12-mer GT duplex between −5 and 62 °C are plotted in Figure 3A. The dG·dC imino resonance at 13.26 ppm broadened out by 5 °C (Figure 3A) and is followed by broadening of the dG·dC imino resonance at 13.21 ppm and both imino protons in the dG·dT interaction at 11.78 and 10.58 ppm at 35 °C (Figures 2 and 3A). Finally, with the onset of the melting transition of the 12-mer GT duplex, the remaining dG·dC imino proton at 12.97 ppm and the two dA·dT imino protons at 13.82 ppm broaden significantly at 47 °C (Figure 2).

[1] Abbreviations: OD, optical density; EDTA, ethylenediaminetetraacetic acid.

Table I: Chemical Shifts of the Imino Exchangeable Protons of the 12-mer GC Duplex[a] and the 12-mer GT Duplex[b] in 0.1 M Phosphate and 2.5 mM EDTA Solution

resonance	assignment[c]	chemical shifts at −5 °C (ppm) 12-mer GC	12-mer GT
G(H-1)	1	13.29	13.26
G(H-1)	2	13.14	13.21
G(H-1)	3	12.96	10.58[d]
G(H-1)	4	12.74	12.97
T(H-3)	5	13.97	13.82
T(H-3)	6	13.86	13.82

[a] pH 7.50. [b] pH 7.70. [c] Guanosine H-1 assignments are based on the sequential broadening of the 12-mer and 13-mer imino exchangeable resonances with increasing temperature. The thymidine H-3 assignments are based on the sequential broadening of the d(GGAATTCC) duplex imino exchangeable resonances with increasing temperature. [d] The thymidine H-3 proton resonates at 11.78 ppm in the dG·dT pair.

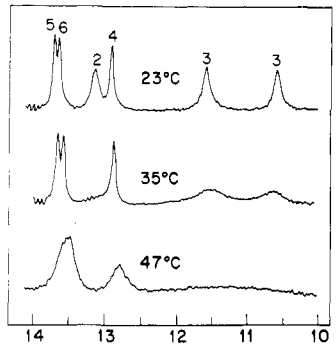


FIGURE 2: The temperature dependence of the 360-MHz correlation proton NMR spectra (10–14 ppm) of the 12-mer GT duplex in 0.1 M phosphate, 2.5 mM EDTA, and 4:1 $H_2O/^2H_2O$, pH 7.70, at 23, 35, and 47 °C. The signal to noise of the spectra was improved by applying a 5-Hz exponential line broadening contribution. The assignments of individual imino protons to specific base pairs in the 12-mer GT sequence are designated above the resonances.

The sequential nature of the broadening of the guanosine H-1 protons with increasing temperature (Figures 2 and 3A) are indicative of fraying at the ends of the duplex (Patel, 1974; Patel & Hilbers, 1975; Kan et al., 1975) and permit the assignment of the imino protons of the dG·dC base pairs (Table I).

The temperature-dependent line widths of the imino proton at guanosine residue 3 in the 12-mer GC and 12-mer GT duplexes in 0.1 M phosphate at pH ~7.6 are plotted in Figure 3B. It is readily apparent that there is considerable destabilization of the 12-mer GT duplex compared to the 12-mer GC duplex as reflected by the exchange characteristics of the corresponding guanosine imino proton at position 3 in the sequence.

(*B*) *Imino Proton Chemical Shifts.* We observe the imino protons from dG·dC base pairs 2 and 4 in the 12-mer GT duplex below 30 °C (Table I, Figure 1), demonstrating that the base pairs are intact adjacent to the dG·dT interaction site at low temperature.

A comparison of the five Watson–Crick imino proton chemical shifts common to the 12-mer GC and 12-mer GT duplexes at −5 °C (Table I) demonstrates that the imino protons at positions 1, 2, and 6 exhibit chemical shift differences of <0.1 ppm while the imino proton of dG·dC base pair 4 shifts downfield by 0.23 ppm and the imino proton of dA·dT base pair 5 shifts upfield by 0.15 ppm (Table I). Thus, the replacement of the dG·dC base pair by a dG·dT interaction at position 3 perturbs the imino proton chemical shift of base pair 4 and to a lesser extent base pair 5 in the sequence.

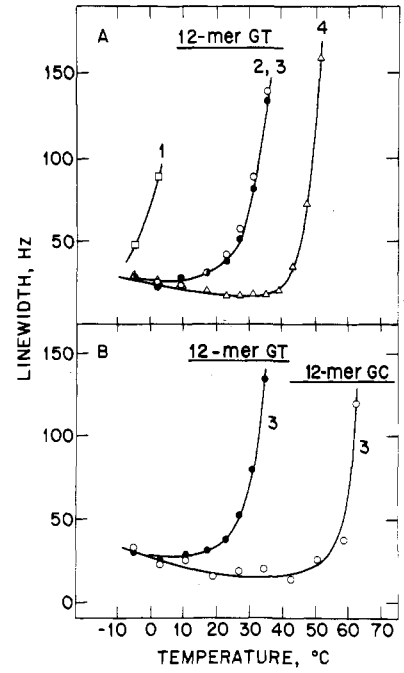


FIGURE 3: (A) The temperature dependence of the line widths of the four dG·dC imino protons of the 12-mer GT duplex in 0.1 M phosphate, 2.5 mM EDTA, and 4:1 $H_2O/^2H_2O$ at pH 7.70. (B) The temperature dependence of the line widths of the imino proton of dG·dC base pair 3 in the 12-mer GC duplex (○) and the 12-mer GT duplex (●) in 0.1 M phosphate, 2.5 mM EDTA, and 4:1 $H_2O/^2H_2O$ at pH 7.50 and 7.70, respectively.

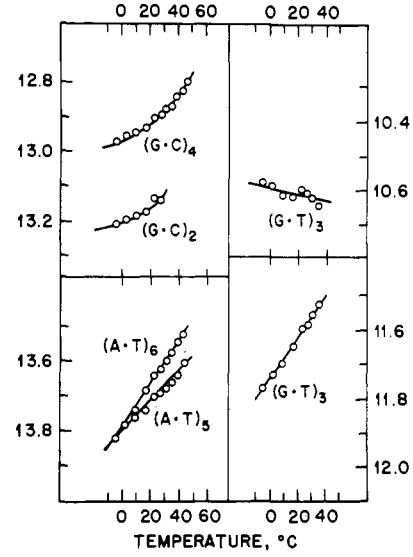


FIGURE 4: The temperature dependence of the nonterminal imino proton chemical shifts in the 12-mer GT duplex in 0.1 M phosphate, 2.5 mM EDTA, and 4:1 $H_2O/^2H_2O$, pH. 7.70, between −5 and 43 °C.

The guanosine H-1 imino resonance of dG·dC base pair 3 at 12.96 ppm in the 12-mer GC duplex shifts upfield to imino resonances at 11.78 and 10.58 ppm for the dG·dT interaction in the 12-mer GT duplex at −5 °C (Table I). We tentatively assign the 11.78 ppm resonance to the thymidine imino proton and the 10.58 ppm resonance to the guanosine imino proton in the dG·dT interaction, based on the lower intrinsic shift of the imino proton of a dA·dT base pair compared to a dG·dC base pair (Hilbers, 1979).

The temperature-dependent chemical shifts of the imino protons of the five nonterminal base pairs in the 12-mer GT duplex between −5 and 43 °C are plotted in Figure 4. We observe large temperature-dependent chemical shifts for the

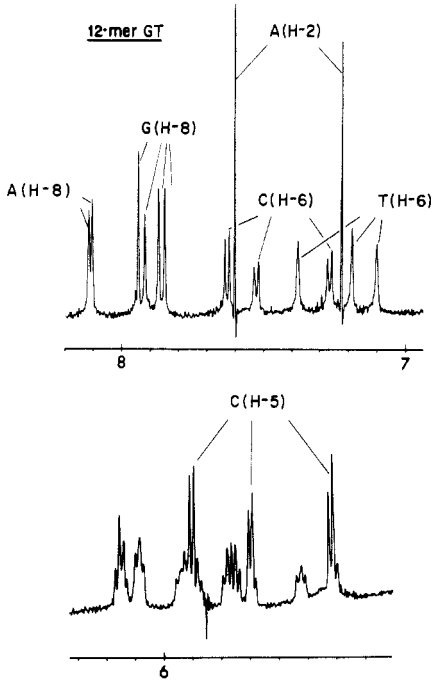


FIGURE 5: The 500-MHz Fourier transform proton NMR spectra (5.2–8.2 ppm) of the 12-mer GT duplex in 0.1 M phosphate, 2.5 mM EDTA, and ^2H_2O at ambient temperature. The resolution of the spectrum was improved by applying an exponential line narrowing contribution.

imino protons of dA·dT base pairs 5 and 6 in the 12-mer GT duplex (Figure 4) similar to what has been observed for the 12-mer GC duplex (Patel et al., 1981). A temperature-dependent chemical shift of the same magnitude is observed at the thymidine H-3 proton of the dG·dT pair at position 3 of the 12-mer GT duplex (Figure 4). By contrast, we observe smaller temperature-dependent chemical shift changes at the guanosine H-1 protons in the 12-mer GT duplex between −5 and 43 °C with almost no change at the dG·dT wobble pair at position 3 and an 0.2 ppm change at dG·dC Watson–Crick base pair at position 4 (Figure 4).

(*C*) *Nuclear Overhauser Effect.* When two protons on different segments of a nucleic acid duplex are within 3.5 Å of each other, it is possible to observe a nuclear Overhauser effect (NOE) between them. Thus, saturating one of the protons perturbs the intensity of its spatially related neighbor with the result readily observable in difference spectra with the irradiation off and on the resonance of interest (Bothner-By, 1979).

We have probed for an NOE effect between the two imino protons in the dG·dT interaction in the 12-mer GT duplex in 0.1 M phosphate and H_2O, pH 5.8, at 1 °C. These experiments were guided by an earlier demonstration of an NOE between the imino protons in the G·U pair in transfer RNA (Johnston & Redfield, 1979).

The saturation of the dG·dT thymidine imino proton at ~11.6 ppm in the 12-mer GT spectrum (Figure 1B) results in the observation of a 15% negative NOE in the difference spectrum at the dG·dT guanosine imino proton at ~10.6 ppm (Figure 1C). Similarly, a 17% negative NOE at the thymidine imino proton at ~11.6 ppm is observed on saturation of the 10.6 ppm guanosine imino proton. These NOE results demonstrate that the imino protons are within 2.6 Å of each other and are consistent with wobble base pair formation involving adjacent imino proton–carbonyl hydrogen bonds (Chart II).

(*D*) *Nonexchangeable Proton Spectra.* The ambient-temperature 500-MHz proton NMR spectrum of the base proton region (5.2–8.2 ppm) of the 12-mer GT duplex in 0.1 M phosphate is presented in Figure 5. The assignments of several of these resonances to specific base pairs is based on spin decoupling, nuclear Overhauser effect, and chemical modification studies on the related 12-mer GC duplex (Patel et al., 1981).

Chart II

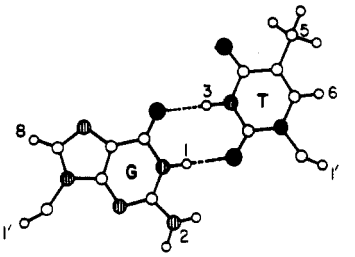


Table II: Chemical Shift Parameters at the dA-dT Base Pairs for the 12-mer GT Duplex in 0.1 M Phosphate Solution[a]

resonance	assignment[b]	chemical shifts (ppm)	T_m (°C)
A(H-8)		8.09	
A(H-8)		8.09	52.0
A(H-2)	5	7.25	
A(H-2)	6	7.61	
T(H-6)	6	7.08	51.0
T(H-6)	5	7.37	52.0
T(CH_3-5)	6		
T(CH_3-5)	5	1.545	51.0

[a] Duplex state at 30 °C. [b] Specific base pair assignments are based on chemical modification and nuclear Overhauser effect measurements on 12-mer and related analogues.

Table III: Chemical Shift Parameters at the dG·dC Base Pairs for the 12-mer GT Duplex in 0.1 M Phosphate Solution[a]

resonance	assignment[b]	chemical shifts (ppm)	T_m (°C)
G(H-8)		7.83	
G(H-8)		7.86	
G(H-8)		7.90	
G(H-8)		7.93	
C(H-6)	2	7.27	51.0
C(H-6)	4	7.51	56.0
C(H-6)	1	7.62	
C(H-5)	2	5.42	
C(H-5)	4	5.70	
C(H-5)	1	5.90	

[a] Chemical shift at 30 °C. [b] Specific resonance assignments are based on spin decoupling and comparison with chemical shifts of $(dC\text{-}dG)_3$ hexanucleotide duplex.

The proton chemical shifts of the two dA·dT base pairs in the 12-mer GT duplex at 30 °C are presented in Table II with the corresponding proton data for the dG·dC base pairs summarized in Table III. In addition, we list the corresponding transition midpoints at individual resonances of the 12-mer GT in 0.1 M phosphate in Tables II and III.

(*E*) *dG·dT Interaction.* The hexanucleotide core contains base pairs 4–6 that are common to the 12-mer GC duplex and the 12-mer GT duplex. This common core results in similar conformational properties and NMR parameters for the thymidines at residues 5 and 6 (Table II). There is an additional thymidine at position 3 in the 12-mer GT duplex, which may be identified by its distinct NMR parameters during the melting transition. The three broad thymidine H-6 singlets in the 12-mer GT duplex are well resolved in the 500-MHz NMR spectrum between 7.1 and 7.4 ppm at ambient temperature (Figure 5) and can be independently

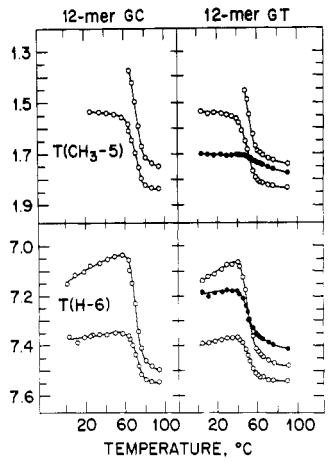


FIGURE 6: The temperature dependence of the thymidine H-6 and CH_3-5 resonances in the 12-mer GC duplex and the 12-mer GT duplex in 0.1 M phosphate, 2.5 mM EDTA, and ^2H_2O. The thymidine resonances at positions 5 and 6 in the 12-mer GC and 12-mer GT duplexes are represented by (○) while the thymidine resonances at position 3 in the 12-mer GT duplex are represented by (●).

Table IV: Chemical Shift Parameters for Thymidine in the dG·dT Interaction in the 12-mer GT Duplex in 0.1 M Phosphate Solution

resonance	chemical shift ppm[a]	chemical shift ppm[b]	T_m (°C)
T(CH_3-5)	1.70	0.07	
T(H-6)	7.18	0.24	52.5

[a] Chemical shift in the duplex state at 30 °C. [b] Chemical shift difference between 95 and 30 °C.

monitored through the melting transition.

The temperature dependence of the thymidine H-6 and CH_3-5 chemical shifts through the melting transitions of the 12-mer GC and 12-mer GT duplexes in 0.1 M phosphate is plotted in Figure 6. The extra thymidine H-6 and CH_3-5 resonances associated with the dG·dT interaction in the 12-mer GT duplex can be readily identified based on its unique chemical shift in the duplex and strand states (Figure 6).

The NMR parameters for the thymidine base protons in the dG·dT pair during the melting transition of the 12-mer GT duplex are tabulated in Table IV. We observe upfield shifts of 0.24 ppm at the H-6 and 0.07 ppm at the CH_3-5 protons at thymidine residue 3 on 12-mer GT duplex formation (Table IV), which are smaller than the upfield shifts of ~0.4 and ~0.5 ppm at the H-6 and H-5 protons of the cytidine residue 3 on 12-mer GC duplex formation (Patel et al., 1981).

(F) Phosphorus Chemical Shifts. Proton noise decoupled 81-MHz ^{31}P spectra of the 12-mer GT duplex in 20 mM phosphate at 18.5 and 62 °C are presented in Figure 7. We observe eight resolved, single phosphorus resonances and an unresolved envelope of three phosphorus resonances between 3.84 and 4.68 ppm upfield from standard trimethyl phosphate at 18.5 °C (Figure 7). The phosphorus chemical shifts are dispersed over an 0.84-ppm region in the duplex state at 18.5 °C and may be compared with a chemical shift dispersion of 0.26 ppm in the strand state at 62 °C (Figure 7).

The 81-MHz ^{31}P spin–lattice relaxation times (T_1) and the $^{31}P[^1H]$ nuclear Overhauser effect ($1 + \eta$) values for the resolved phosphodiesters in the 12-mer GT duplex at 20 °C are summarized in Table V.

Calorimetry. The derived heat capacity curve for the 12-mer GT duplex in 0.1 M NaCl is shown in Figure 8. For comparison, the corresponding curve for the 12-mer GC duplex is also included (Patel et al., 1981). The transition midpoints are 71.3 °C for the 12-mer GC and 51.3 °C for the 12-mer GT (Table VI).

(A) Calorimetric Enthalpy. From the areas under the heat capacity curves (Figure 8), we obtain calorimetric enthalpies

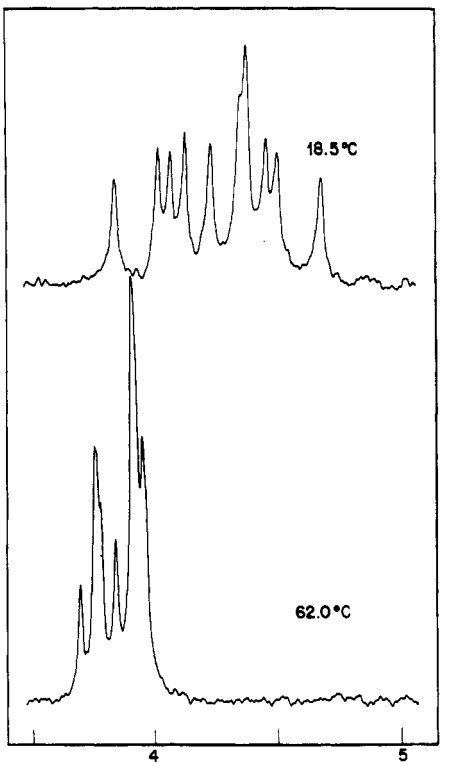


FIGURE 7: The proton noise decoupled 80.995-MHz Fourier transform phosphorus NMR spectra (3.5–5.0 ppm upfield from standard trimethyl phosphate) of the 12-mer GT duplex in 20 mM phosphate, 0.5 mM EDTA, and ^2H_2O, pH 7.88, at 18.5 and 62 °C. The chemical shifts are not corrected for the temperature dependence of the standard.

Table V: 81-MHz ^{31}P Spin–Lattice Relaxation Time (T_1) and Nuclear Overhauser Effect ($1 + \eta$) for the Resolved Phosphodiesters in the 12-mer GT Duplex at 20 °C[a]

chemical shift (ppm)[b]	area	relaxation time, T_1 (s)[c]	NOE $(1 + \eta)$[c]
3.827	1	1.86	1.09
4.006	1	1.79	1.05
4.057	1	1.77	1.05
4.120	1	1.90	1.06
4.219	1	1.53	1.09
4.336[d]	1	1.72	
4.362	2	1.67	1.02
4.446	1	1.50	0.99
4.490	1	1.51	1.02
4.680	1	1.61	1.04

[a] Buffer: 20 mM phosphate, 0.5 mM EDTA, and ^2H_2O, pH 7.1. [b] Upfield from internal standard trimethyl phosphate. [c] The repetition delay between pulses was 10 s. [d] Shoulder on 4.362 ppm resonance.

Table VI: Calorimetric and van't Hoff Enthalpies for the Melting Transition of the 12-mer GC and the 12-mer GT Duplexes in 0.1 M NaCl Solution[a]

	ΔH_{cal} (kcal)	$\Delta H_{V.H.}$ (kcal)	T_m (°C)
12-mer GC	102	74	71.3
12-mer GT	106	76	51.3

[a] Buffer: 0.1 M NaCl, 10 mM phosphate, and 0.1 mM EDTA, pH 7.0. Calorimetric data represent averages of at least three independent determinations.

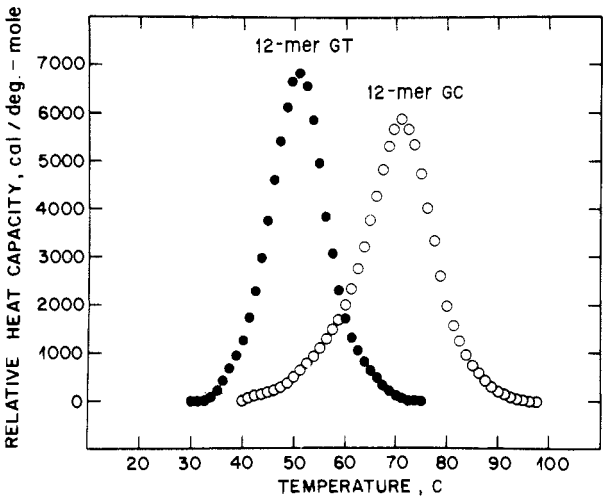


FIGURE 8: Calorimetric heat capacity vs. temperature curves for the 12-mer GC duplex (strand concentration 0.65 mM) and the 12-mer GT duplex (strand concentration 0.70 mM) in 0.1 M NaCl, 10 mM phosphate, and 0.1 mM EDTA, pH 7.0. The temperature was scanned from 10 to 95 °C at a rate of ~1 °C/min.

of 106 kcal for the order–disorder transition of the 12-mer GT duplex and 102 kcal for the 12-mer GC duplex (Patel et al., 1981) (Table VI). Within the limits of the calorimetric measurement, these two transition enthalpies are essentially indistinguishable. Thus, despite a 20 °C drop in T_m, substitution of two dG·dC base pairs by two dG·dT base pairs at the third position from each end does not significantly alter the enthalpy of the duplex to single-strand transition.

(B) van't Hoff Enthalpy. As described in the previous paper, analysis of the shapes of the calorimetric heat capacity curves (Figure 8) allows calculation of the van't Hoff enthalpy changes. Essentially equal transition enthalpies of 76 kcal for the 12-mer GT duplex and 74 kcal for the 12-mer GC duplex (Patel et al., 1981) are obtained (Table VI). Thus, for both duplexes the model-dependent van't Hoff enthalpies are substantially lower in magnitude than the model-independent calorimetric values (Table VI). The significance of this inequality will be discussed.

Discussion

Wobble Pair Formation. The observed ~16% negative nuclear Overhauser effect between the guanosine and thymidine imino protons in the dG·dT pair at position 3 in the 12-mer GT duplex (Figure 1) is similar in magnitude and sign to that observed for the imino protons of G·U pairs in several tRNAs in solution (Johnston & Redfield, 1979). Therefore, our studies on the 12-mer GT duplex demonstrate that the two imino protons in the dG·dT interaction are in close proximity as required for wobble base pair formation. Furthermore, the guanosine H-1 and thymidine H-3 imino protons in the dG·dT pair at position 3 resonate upfield from the remaining imino protons (Figure 1) due to the formation of hydrogen bonds with carbonyl acceptor groups in the wobble interaction (Crick, 1966; Lezius & Domin, 1973). Our observation of two imino resonances between 10 and 12 ppm rules our alternate pairing proposals containing a single imino proton. These data also exclude hydrogen-bonding interactions involving tautomeric states of the purine and pyrimidine bases.

Fraying at Ends of Duplex. The imino proton of dG·dC base pair 1 in the 12-mer GT duplex broadens out on raising the temperature from −5 to +5 °C at pH 7.7 (Figure 3A). This broadening demonstrates fraying of the terminal base pair in 0.1 M phosphate solution. The imino proton of dG·dC base pair 2 and both imino protons of dG·dT base pair 3 in the 12-mer GT duplex broaden to the same extent on raising the temperature to 35 °C (Figures 2 and 3A), consistent with the fraying process extending to base pair 3 in the interior of the dodecanucleotide duplex. By contrast, the broadening is sequential in the 12-mer GC duplex with imino proton of base pair 2 exchanging out before that of base pair 3 (Patel et al., 1981). Thus, we observe a somewhat faster exchange rate from the imino proton in a dG·dT pair relative to the corresponding imino proton in a dG·dC base pair located in the interior of a dodecanucleotide duplex.

Imino Protons in Wobble Pair. The thymidine H-3 proton in the wobble pair shifts significantly to high field with increasing temperature while the guanosine H-1 proton shifts slightly to low field in the same temperature range (Figure 4). The mutual shift of the two imino protons toward each other may imply exchange between the dG·dT imino protons in the 12-mer GT duplex with increasing temperature. We suggest that exchange with solvent occurs through a common pathway since both imino protons in the wobble pair of the 12-mer GT duplex broaden to the same extent with increasing temperature (Figure 2). These results are consistent with an earlier proposal that base pair opening is a prerequisite for exchange of imino protons in oligonucleotide duplexes (Mandal et al., 1979).

Mismatch Site. The thymidine proton markers on residue 3 shift upfield to a smaller extent on 12-mer GT duplex formation (Table IV) compared to the corresponding markers on the cytidine residue at the same position on 12-mer GC duplex formation (Patel et al., 1981). This demonstrates that in these dodecanucleotide duplexes dG·dC base pairs 2 and 4 stack to a lesser degree with the thymidine in the dG·dT wobble pair compared to the cytidine in the dG·dC Watson–Crick pair. We are unable to correlate the guanosine H-8 proton at position 3 in the 12-mer GC and 12-mer GT duplexes and are, hence, unable to unravel the stacking arrangement of this purine with adjacent base pairs at the mismatch site.

The phosphorus spectra of the 12-mer GT duplex at 18.5 °C displays single resonances at 3.84 and 4.68 ppm on either side of the 4.0–4.5-ppm cluster characteristic of oligonucleotide duplexes (Figure 7). These two resonances, which are separated by 0.84 ppm, are tentatively assigned to the phosphodiesters at the wobble base pair site in the 12-mer GT duplex.

Base Pairs Adjacent to Wobble Pair. The low-temperature imino proton NMR data (Table I, Figure 1) reveal that base pairing is maintained on either side of the wobble pair. Nevertheless, formation of the dG·dT pair results in changes in base pair overlaps and phosphodiester torsion angle changes at the mismatch site. Specifically, we observe chemical shift differences at the guanosine H-1 of base pair 4 between the 12-mer GC and 12-mer GT duplexes (Table I). Furthermore, the imino proton of dG-dC base pair 4 in the 12-mer GT duplex exhibits a temperature-dependent chemical shift change (Figure 4) that is much larger than the corresponding change at this position in the 12-mer GC duplex (Patel et al., 1981). These data imply wobble base pair formation at position 3 results in a perturbation of the adjacent dG·dC base pair 4.

By contrast, we observe similar proton chemical shifts for the base resonances in the 12-mer GC and 12-mer GT duplexes at the dA·dT residues 5 and 6 and dG·dC residue 1, which are distant from the mismatch site. This demonstrates the ability of the DNA duplex to localize the conformational change associated with the incorporation of a dG·dT mismatch in the interior of the duplex.

Phosphodiester Backbone. The resolution of 8 of the 11 phosphodiesters in the 12-mer GT duplex over an 0.84-ppm

chemical shift dispersion at 18.5 °C (Figure 7) contrasts strikingly with the smaller dispersion observed in previous studies of deoxyoligonucleotide duplexes (Patel, 1976; Davanloo et al., 1979; Patel & Canuel, 1979). This suggests that the introduction of the symmetry-related wobble pairs at position 3 in the dodecanucleotide duplex perturbs the phosphodiester conformation at the O–P–O bond angles and/or O–P torsion angles (Patel, 1976; Gorenstein & Kar, 1977; Simpson & Shindo, 1980), resulting in a well-resolved phosphorus spectrum for the 12-mer GT duplex. Since the phosphorus resonances cannot be definitively assigned to specific phosphodiesters in the 12-mer GT sequence, further discussion would be speculative at this time.

The spin–lattice relaxation times (T_1) and the nuclear Overhauser effect ($1 + \eta$) values for the two shifted phosphorus resonances at 3.83 and 4.68 ppm are similar to the remaining resonances between 4.0 and 4.5 ppm in the 12-mer GT duplex at 20 °C (Table V). Thus, the chemical shift is a more sensitive indicator than the relaxation parameters for studying the conformational perturbation associated with these resonances that have been tentatively assigned to the phosphodiesters in the wobble site in the 12-mer GT duplex.

Melting Transition. The melting transition of the 12-mer GT duplex has been monitored at the nonexchangeable protons of dA·dT base pairs 5 and 6 (Table II), dG·dC base pairs 2 and 4 (Table III), and dG·dT wobble pair 3 (Table IV). These markers distributed among the nonterminal base pairs in the helix exhibit a common transition midpoint of 52 °C in 0.1 M phosphate solution, which demonstrates that the 10 nonterminal base pairs of the dodecanucleotide melt as a single helix during the duplex to strand transition. We are unable to evaluate melting transition midpoints from the small chemical shifts between duplex and strand states at the terminal base pairs.

Overall Stability. Inspection of the melting temperature data indicates that at comparable concentrations NMR (Figure 6) and DSC (Figure 8) provide essentially equivalent means for determining T_m values for the cooperative transitions of the 12-mer GC and 12-mer GT duplexes. Both techniques reveal that replacement of two dG·dC by two dG·dT base pairs causes approximately a 20 °C drop in the transition midpoint.

Enthalpy Change. Inspection of the calorimetric data in Table VI reveals that despite the 20 °C GT-induced destabilization of the 12-mer GT duplex relative to its GC counterpart, the transition enthalpies are essentially equal. This implies that the two wobble dG·dT base pairs in the 12-mer GT duplex do not significantly disrupt the base stacking interactions relative to those that exist in the 12-mer GC duplex. This conclusion is supported by the NMR data, which reveals that the dG·dT base pairs, like the dG·dC pairs they replace, stack in the helix and that only minor, local perturbations occur in the base stacking of neighboring base pairs.

Since the T_m values for two duplexes differ by 20 °C despite similar transition enthalpies, we conclude that the destabilization of the 12-mer GT relative to the 12-mer GC is entropic in origin. One possible explanation would envision more severe geomtric constraints for the wobble dG·dT base pairing interaction than for the Watson–Crick dG·dC interaction. This would result in a lowering of the entropy of the 12-mer GT duplex relative to the 12-mer GC duplex. As a consequence, a larger entropy change would favor disruption of the 12-mer GT duplex resulting in the observed lower melting temperature.

One note of caution should be mentioned concerning the argument presented above. A 20 °C change in T_m corresponds to a free energy difference between duplexes of only a few kilocalories, which falls within the uncertainty associated with a DSC measurement on a transition involving 100 kcal. Even though multiple DSC runs reduced the average deviation to between 3 and 5%, we cannot eliminate the possibility that part of the observed 20 °C destabilization is enthalpic in origin. However, this would require that the uncertainties in both measurements manifest themselves completely and in opposite directions. Therefore, we have suggested that the GT-induced destabilization is entropic in origin and have presented above a reasonable physical picture as a basis for further discussion.

Since the two duplexes have similar transition enthalpies, the observed differences in phosphodiester torsion angles (as noted by ^{31}P chemical shift data) must not be accompanied by a significant enthalpy effect. This is consistent with the results of a recent NMR and calorimetric study of salt-induced backbone changes in poly(dA-dT) (Marky et al., 1981b).

The conclusions reached above implicitly involve two very reasonable assumptions: namely, that the high-temperature, single-stranded states of the two oligomers are approximately isoenergetic and that no fortuitous compensation of thermal effects is occurring. The high-temperature NMR data support the former assumption.

Finally, inspection of Figure 8 reveals that the heat capacities of the initial and final states for each transition are approximately equal. Thus, we can conclude that the ΔC_p (change in heat capacity at constant pressure) for both transitions is close to zero. This conclusion is consistent with recent calorimetric studies on the melting transitions of other deoxyoligonucleotides (Marky et al., 1981a; Albergo et al., 1981).

Nature of the Transition. As described in the previous paper, the calorimetric data provide a means for testing whether the transition occurs in a highly cooperative, two-state manner ($\Delta H_{cal} = \Delta H_{V,H}$) or if intermediate states are significantly populated ($\Delta H_{cal} > \Delta H_{V,H}$) (Tsong et al., 1970).

Inspection of the data in Table VI reveals that the van't Hoff enthalpies for both transitions are considerably smaller in magnitude than the calorimetrically determined values. Thus, we may conclude that the helix-to-coil transitions for both the 12-mer GC and 12-mer GT do not occur in all all-or-none manner but rather involve partial opening of the helix prior to the cooperative component of the transition. Finally, it should be noted that the size of the cooperative melting unit, as determined from the ratio of the van't Hoff and calorimetric enthalpies, remains unchanged at 9 ± 1 base pairs despite the wobble base pair induced destabilization of the 12-mer GT duplex relative to the 12-mer GC duplex.

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References

Albergo, D., Marky L., Breslauer, K., & Turner, D. (1981) *Biochemistry 20*, 1409–1413.

Arnott, S. (1977) *1st Cleveland Symposium Macromolecules* pp 87–104, Elsevier, Amsterdam.

Arnott, S., Chandrasekaran, R., & Selsing, E. (1975) in *Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions* (Sundaralingam, M., & Rao, S. T., Eds.) pp 577–596, University Park Press, Baltimore, MD.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I., Jr. (1974) *Physical Chemistry of Nucleic Acids*, Harper and Row, New York.

Borer, P. N., Dengler, B., & Tinoco, I., Jr. (1974) *J. Mol. Biol. 86*, 843–853.

Bothner-By, A. A. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., Ed.) pp 177–219, Academic Press, New York.

Breslauer, K. J., Sturtevant, J. M., & Tinoco, I., Jr. (1975) *J. Mol. Biol. 99*, 549–565.

Burd, J. F., Larson, J. E., & Wells, R. D. (1975) *J. Biol. Chem. 250*, 6002–6007.

Cantor, C., & Warshaw, M. W. (1970) *Biopolymers 9*, 1059–1077.

Cornelis, A. G., Haasnoot, J. H. J., den Hartog, J. F., de Rooij, M., van Boom, J. H., & Altona, C. (1979) *Nature (London) 281*, 235–236.

Craig, M. E., Crothers, D. M., & Doty, P. M. (1971) *J. Mol. Biol. 62*, 383–401.

Crick, F. H. C. (1966) *J. Mol. Biol. 19*, 548–555.

Davanloo, P., Armitage, I. M., & Crothers, D. M. (1979) *Biopolymers 18*, 663–680.

Dodgson, J. B., & Wells, R. D. (1977) *Biochemistry 16*, 2367–2374.

Early, T. A., Kearns, D. R., Burd, J. F., Larson, J. E., & Wells, R. D. (1977) *Biochemistry 16*, 541–551.

Early, T. A., Olmsted, J., III, Kearns, D. R., & Lezius, A. G. (1978) *Nucleic Acids Res. 5*, 1955–1970.

Gillam, S., Waterman, K., & Smith, M. (1975) *Nucleic Acids Res. 2*, 625–634.

Gorenstein, D. G., & Kar, D. (1977) *J. Am. Chem. Soc. 99*, 672–677.

Gralla, J., & Crothers, D. M. (1973) *J. Mol. Biol. 73*, 497–511.

Haasnoot, C. A. G., den Hartog, J. H. J., deRooij, J. F., van Boom, J. H., & Altona, C. (1980) *Nucleic Acids Res. 8*, 169–181.

Hilbers, C. W. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., Ed.) pp 1–44, Academic Press, New York.

Hirose, T., Crea, R., & Itakura, K. (1978) *Tetrahedron Lett. 28*, 2449–2452.

Johnston, P. D., & Redfield, A. G. (1979) in *Transfer RNA, Structure, Function, and Recognition* (Schimmel, P., Abelson, J., & Soll, D., Ed.) pp 191–206, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kallenbach, N. R., Daniel, W. E., Jr., & Kaminker, M. A. (1976) *Biochemistry 15*, 1218–1224.

Kan, L. S., Borer, P. N., & T'so, P. O. P. (1975) *Biochemistry 14*, 4864–4869.

Kearns, D. R., Patel, D. J., & Shulman, R. G. (1971) *Nature (London) 229*, 338–339.

Leslie, A. G. W., Arnott, S., Chandrasekaran, R., & Ratliff, R. L. (1980) *J. Mol. Biol. 143*, 49–72.

Łezius, A. G., & Domin, E. (1973) *Nature (London) 244*, 169–170.

Mandal, C., Kallenbach, N. R., & Englander, S. W. (1979) *J. Mol. Biol. 135*, 391–411.

Marky, L. A., Canuel, L., Jones, R. A., & Breslauer, K. J. (1981a) *Biophys. J. 13*, 141–149.

Marky, L. A., Patel, D. J., & Breslauer, K. J. (1981b) *Biochemistry 20*, 1427–1431.

Martin, F. H., Uhlenbeck, O. C., & Doty, P. (1971) *J. Mol. Biol. 57*, 201–215.

Patel, D. J. (1974) *Biochemistry 13*, 2396–2402.

Patel, D. J. (1976) *Biopolymers 15*, 533–558.

Patel, D. J., & Hilbers, C. W. (1975) *Biochemistry 14*, 2656–2660.

Patel, D. J., & Canuel, L. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 674–678.

Patel, D. J., & Canuel, L. L. (1979) *Eur. J. Biochem. 96*, 267–276.

Patel, D. J., Kozlowski, S. A., Marky, L. A., Broka, C., Rice, J. A., Itakura, K., & Breslauer, K. J. (1981) *Biochemistry* (first paper of four in this issue).

Porschke, D., Uhlenbeck, O. C., & Martin, F. H. (1973) *Biopolymers 12*, 1313–1335.

Schimmel, P. R., & Redfield, A. G. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 181–221.

Selsing, E., & Wells, R. D. (1979) *J. Biol. Chem. 254*, 5410–5416.

Selsing, E., Wells, R. D., Early, T. A., & Kearns, D. R. (1978) *Nature (London) 275*, 249–250.

Simpson, R. T., & Shindo, H. (1980) *Nucleic Acids Res. 8*, 2093–2103.

Topal, M., & Fresco, J. R. (1976) *Nature (London) 263*, 285–293.

Tsong, T. Y., Hearn, R. P., Wrathall, D. P., & Sturtevant, J. M. (1970) *Biochemistry 9*, 2666–2677.

Uhlenbeck, O. C., Martin, F. H., & Doty, P. (1971) *J. Mol. Biol. 57*, 217–229.

Uhlenbeck, O. C., Borer, P. N., Dengler, B., & Tinoco, I., Jr. (1973) *J. Mol. Biol. 73*, 483–496.

Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., & Itakura, K. (1979) *Nucleic Acids Res. 6*, 3543–3557.

Wells, R. D., & Wartell, R. M. (1974) in *Biochemistry of Nucleic Acids* (Burton, K., Ed.) Vol. 6, pp 41–64, Butterworth, London, England.

Wells, R. D., Blakesley, R. W., Hardies, S. C., Horn, G. T., Larson, J. E., Selsing, E., Burd, J. F., Chan, H. W., Dodgson, J. B., Nes, I. F., & Wartrell, R. M. (1977) *CRC Crit. Rev. Biochem. 4*, 305–340.

Young, P. R., & Kallenbach, N. R. (1978) *J. Mol. Biol. 126*, 467–479.