Nuclear Magnetic Resonance Study of Hydrogen-Bonded Ring Protons in Oligonucleotide Helices Involving Classical and Nonclassical Base Pairs†

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ABSTRACT: A study of the exchangeable ring nitrogen protons in aqueous solutions of oligonucleotide complexes involving Watson–Crick base pairs as well as Hoogsteen pairs and other nonclassical hydrogen bonding schemes shows that resolvable resonances in the low-field (−10 to −16 ppm from sodium 4,4-dimethyl-4-silapentanesulfonate) region can be detected in a variety of structures other than double stranded helices. Ring nitrogen proton resonances arising from the following hydrogen-bonding situations are reported: (1) AT and GC Watson–Crick base pairs in a self-complementary octanucleotide, dApApApGpCpTpT-pT; (2) U·A·U base triples in complexes between oligo-U_{15} and AMP; (3) C·G·C^+ base triples in complexes between oligo-C_{17} and GMP at acid pH; (4) s^4U·A·s^4U base triples in complexes between oligo-s^4U_{15} and AMP, all of which involve both Watson–Crick and Hoogsteen base pairing to form triplexes; (5) C·C^+ base pairing between protonated and unprotonated C residues in oligo-C_{17} at acid pH; and (6) I_4 base quadruples in the four strand association among oligo-I at high salt. The behavior of the $dA_3G{\cdot}CT_3$ helix is consistent with both fraying of the terminal base pairs and presence of intermediate states as the helix opens. In the monomer–oligomer complexes, under the conditions used here, the exchange appears to be governed by the dissociation rate of monomer from the complex. These findings suggest that those tertiary structure hydrogen bonds in tRNA involving ring nitrogen protons should have representative resonances in the low-field (11–16 ppm) proton NMR region in H_2O.

At low temperatures, both the ring nitrogen protons and exocyclic amino protons of bases participating in helical nucleic acid structures can be observed to exchange slowly with solvent water protons (Englander et al., 1972). Slow exchange of the ring N_1-H of guanine and N_3-H of uracil or thymine, when involved in the hydrogen bonds of base pairs, has been shown to produce characteristic low-field resonances (10 to 15 ppm downfield from a DSS[1] standard) in the proton NMR spectra of tRNA (Kearns and Shulman, 1974), as well as a number of model helical oligonucleotide duplexes (Crothers et al., 1973; Patel and Tonelli, 1974) in H_2O. Provided the molecules are capable of sufficiently rapid rotational tumbling, high resolution proton NMR can be used to assess both the nature and stability of hydrogen-bonded nucleic acids in aqueous solution (Crothers et al., 1974; Patel and Hilbers, 1975; Hilbers and Patel, 1975). The frequency of the resonance of the ring nitrogen proton in a particular base pair of a helix has been accounted for in terms of shielding exerted by the ring currents of nearest neighbor bases in the helix (Shulman et al., 1973; Patel and Tonelli, 1974). For simple double helical structures, the chemical shifts of the ring nitrogen protons reflect the sequence of base pairs present.

Recent models of the three-dimensional structure of crystalline yeast $tRNA^{Phe}$ include three base triples among the tertiary interactions in this molecule (Robertus et al., 1974; Kim et al., 1974). It appears from solution studies of the proton NMR of spin-labeled *E. coli* $tRNA_f^{Met}$ that these triples may be common to all class I tRNA's (Daniel and Cohn, 1975, and unpublished results). This feature has prompted us to investigate the proton NMR spectra of nonclassical base pairs and higher associations in aqueous solution to determine if they can in fact give rise to resonances in the low-field region. We report here the low-field proton NMR spectra of a self-complementary octanucleotide dApApApGpCpTpTpT, capable only of Watson–Crick pairing, as a reference for the behavior of these standard base pairs. We then present spectra of synthetic oligoribonucleotide complexes involving nonstandard associations: (1) the base triple U·A·U; (2) the triple C·G·C^+; (3) the triple s^4U·A·s^4U; (4) the acid C·C^+ hemiprotonated pair; and (5) the I_4 base quadruple. The first three of these are formed by mixing purine monomers, AMP or GMP, with pyrimidine oligomers, U_N, C_N, or s^4U_N of mean chain length N about 15 residues. The A·U_2 triple involves both a standard Watson–Crick pair between adenine and one of the uracil strands, and a Hoogsteen (1959) type of pair linking the second uracil to adenine (Arnott and Bond, 1973; Figure 1A) Protonation of one of the C residues permits formation of a similar triple involving one Watson–Crick G·C pair with a second protonated C connected by means of a Hoogsteen pair as illustrated in Figure 1B. Fiber diffraction studies of this complex have not been reported, although Arnott and Bond (1973) present a structure isomorphic to the U·A·U model for this triple, and infrared spectroscopy of the monomer–polymer complex is consistent with such a model (Howard et al., 1964). The acid oligo-C helical structure (Figure 1C) determined by fiber diffraction analysis on poly(C) (Langridge and Rich, 1963) involves two parallel strands, with one base protonated in each pair. Finally the high salt oligo-I structure involving three (Davies and Rich, 1958) or, more likely, four I strands as shown in Figure 1D (Zimmerman et al., 1975; Arnott et al., 1974)

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[1] Abbreviations used: DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; Mes, 4-morpholinoethanesulfonic acid.

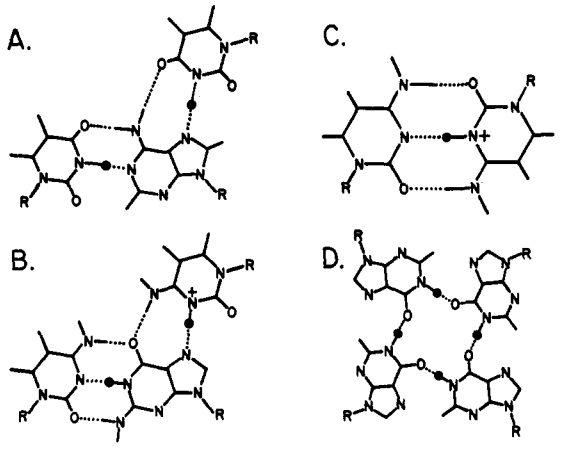

FIGURE 1: Structures determined or proposed for the non-Watson-Crick hydrogen-bonded complexes reported here. (A) The U·A·U triplet determined from fiber diffraction of poly U·poly U (Arnott and Bond, 1973). (B) Proposed C·G·C^+ triplet isomorphic to the poly(U·poly(A)·poly(U) helix (Arnott and Bond, 1973). (C) The C·C^+ hemiprotonated pairing in acid poly(C) (Langridge and Rich, 1963). (D) Proposed four-helix structure for poly(I) in high salt isomorphic with the GMP or poly(G) helix (Arnott et al. 1974; Zimmerman et al., 1975).

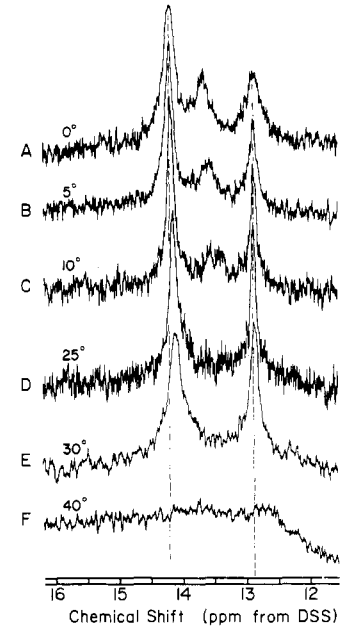

FIGURE 2: Low-field NMR spectrum of the self-complementary deoxyoctanucleotide dA_3GCT_3 (9 mM) in 1.5 M NaCl, 0.01 M Tris-HCl, 5 × 10^{-4} M EDTA, pH 7, at indicated values of the temperatures.

(the fiber diffraction pattern of poly(I) is similar to that of four stranded poly(G)) was prepared by limited alkaline hydrolysis of the polymer. In each case, low-field resonances of the ring protons involved in these nonclassical associations have been detected.

It is thus likely that certain of the nonstandard tertiary hydrogen-bonding interactions should have representative resonances in the low-field (11–15 ppm) proton NMR spectra of tRNA, and that the earlier estimates from NMR of the total number of base pairs present in tRNA should be revised upward from the original values of 19 ± 1 pairs per molecule to 26 or so, in agreement with the figures originally estimated from tritium exchange experiments on tRNA (Englander et al., 1972; Goldstein et al., 1972) and with the estimates from more recent NMR work (Reid et al., 1975; Daniel and Cohn, 1975).

The monomer–oligomer complexes described here represent useful experimental systems for NMR investigation both because the tendency of simple oligomers to aggregate at high concentration is avoided and because the free monomer concentration can be independently varied to control the dissociation rate of the complex. These systems should permit detailed tests of current models for exchange processes in helices.

Materials and Methods

The self-complementary octanucleotide, dApA-pApGpCpTpTpT (dA_3GCT_3), was prepared by Collaborative Research, Inc., Waltham, Mass., under contract with the National Institutes of Health and was made available to us by Dr. F. Bergmann of NIGMS. Characterization of this material by uv spectroscopy (ϵ_{260} 4.5 × 10^4, pH 7), chromatography, enzymatic digestion, and thermal denaturation has been reported by Dr. A. Taunton-Rigby, Collaborative Research. The T_m in 0.01 M Mes buffer, pH 6, with 0.1 M $MgCl_2$ and 0.15 M NaCl present, was determined to be 29 °C for a solution of 11 μM. Denaturation of the sample used for the NMR studies in a buffer containing 1.5 M NaCl, 0.1 M Tris, and 10^{-3} M EDTA was carried out at 301 nm giving an apparent T_m near 50 °C.

Monomer–oligomer complexes were prepared by mixing the components and heating to 60 °C for several minutes. Oligo-U and oligo-C were prepared by limited alkaline hydrolysis of poly(U) and poly(C) (PL Biochemicals) according to the procedure of Bock (1967), followed by chromatography of the hydrolysate on RPC-5 using modification of the conditions described by Egan and Kelmers (1974). Oligo-C fractions of chain length 12–20, mean 17, were obtained from Collaborative Research. Oligo-I was prepared by hydrolysis of the poly(rI) (PL Biochemicals), at pH 10 and 100 °C, and was used without further fractionation. Oligo-s^4U was prepared from fractionated oligo-rC by the amino–thiol exchange reaction (Ueda et al., 1971), carried out in pyridine solution in the presence of H_2S as described by Favre and Fourrey (1974). A reference sample of poly(s^4U) was kindly provided by Professor Y. Lapidot of the Hebrew University of Jerusalem. Monomers AMP and GMP were obtained from Boehringer, N^6-methyladenosine was from Cyclo Chemical, and tubercidin was from Sigma.

NMR spectra were taken on a Varian HR 220-MHz spectrometer operating in the CW mode. Signals were averaged to improve spectral signal-to-noise ratios with a Varian 620i computer. The sample temperature was controlled to ±0.4 °C with a Varian variable-temperature accessory. Chemical shifts reported are with respect to the standard DSS (sodium 4,4-dimethyl-4-silapentanesulfonate).

Results

1. Watson–Crick Base Pairs in dA_3GCT_3. The low-field spectrum of the self-complementary octanucleotide dApA-pApGpCpTpTpT in aqueous solution is shown in Figure 2 for various values of the temperature. The dimeric form of this molecule

A A A G C T T T
| | | | | | | |
T T T C G A A A

forms a palindromic helix of eight residues so that, in principle, four distinct ring proton resonances should be present,

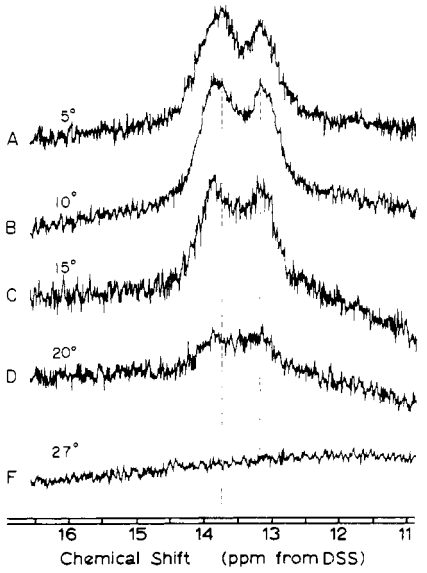

FIGURE 3: Low-field NMR spectrum of a mixture of AMP (100 mM) and oligo-U_{15} (20 mM in U) in 1.5 M NaCl, 0.01 Tris-HCl, 5×10^{-4} M EDTA, pH 7, at indicated values of the temperature.

corresponding to the three T-N_3 protons of the AT Watson-Crick base pairs and the single G-N_1 proton of the G·C Watson-Crick pair. The spectrum in panel A (0 °C) consists of three distinct resonances, at 12.9, 13.7, and 14.2 ppm downfield from an internal DSS standard. From the progression with increasing temperature and the anticipated opening of the helix from the ends, the resonance at 13.7 ppm corresponds to the N_3 proton of T in the terminal A·T pairs of the helix. The initial intensity of the 14.2 ppm peak is twice that of the 12.9 ppm resonance, and this ratio diminishes with increasing temperature. Hence we can assign the 14.2 ppm resonance to the N_3 proton of T in the internal A·T base pairs of the helix, while the stable 12.9 ppm resonance then corresponds to the N_1 proton of G in the internal G·C base pairs. It should be noted that the sequential opening of the helix from the ends is predicted by the standard treatments of helix–coil transitions and favored additionally by the greater thermodynamic stability of the core G·C pairs (Kallenbach, 1968). If we accept the reference positions of 13.6 ± 1 ppm for the N_1-H of T in an isolated A·T pair, the positions for the four resonances in the A_3 G·CT helix can be estimated from the set of ring current shifts for the DNA "B" helix (Patel and Tonelli, 1974) based on the magnetic isoshielding contours predicted according to Giessner-Prettre and Pullman (1970):

T: N_3H	(A·T) terminal (position)	14.31 ppm
T: N_3H	(A–T) position 2	14.12 ppm
T: N_3H	(A–T) position 3	14.18 ppm
G: N_1H	(G·C) position 4	13.08 ppm

The positions of the internal A·T and G·C resonances accord reasonably well with their calculated values, while the terminal A·T resonances do not. Patel and Hilbers (1975) have ascribed this anomaly to fraying of the terminal base pair of the helix, as is discussed more fully below.

2. Watson-Crick and Hoogsteen Base Pairs in the U·A·U Complex. At sufficiently high monomer concentrations, mixtures of monomer adenosine or AMP with poly(U) lead to formation of a fairly stable triple helical complex (Howard et al., 1966). This complex exhibits infrared adsorbtion bands characteristic of the analogous three-stranded helix poly(U·A·U), formed by mixing poly(A) with excess poly(U) at high salt concentration (Miles and Frazier, 1964; Stevens and Felsenfeld, 1964). Moreover both uv and ir mixing experiments indicate that the triple helix U·A·U is the only stable species, even in the presence of excess monomer (Howard et al., 1966; Scruggs and Ross, 1970).

The stability of a monomer·polymer or monomer·oligomer complex is a function of the monomer concentration. Theoretical considerations (Magee et al., 1963; Damle, 1970) dictate a logarithmic relationship between the T_m of such structures and the concentration of monomer. According to Damle (1970), for a mixture of monomers of concentration C_m and oligomer of concentration C_o, the T_m is given by:

$$\frac{1}{T_m(C_m,C_o)} = \frac{\Delta S^\circ}{\Delta H^\circ} + \frac{R}{\Delta H^\circ} \ln \chi \, (C_m - \tfrac{1}{2} C_o)$$

where T_m is the midpoint of the dissociation profile, ΔS° is the standard entropy, ΔH° is the enthalpy of formation of the complex, and χ is a nucleation constant. Since $\Delta H^\circ < 0$, this relation predicts increasing stabilization of the complex as C_m is increased; providing disproportionation does not occur, it is thus advantageous to work with excess monomer. The low-field NMR spectrum of a mixture of heterogeneous U_N oligomers of chain length between 10 and 20 with monomer AMP is shown in Figure 3 for different temperature values. Two resonances of approximately equal intensity are observed, one at 13.8 ppm and the other at 13.2 ppm downfield from DSS. These broaden to similar extents as the temperature increases, finally disappearing at 20 °C, as expected in view of the greatly diminished T_m of monomer–oligomer helices with respect to helices of exclusively polymeric strands (Scruggs and Ross, 1970).

Assuming the complex between AMP and oligo-U_{15}[2] has the 12-fold helical structure of the poly(U)·poly(A)·poly(U) helix determined by x-ray diffraction analysis of fibers (Arnott and Bond, 1973), we can estimate the relative ring current shielding exerted by vicinal bases in the helix on the uracil N_3 proton situated in the Watson-Crick pair and the uracil N_3 proton in the Hoogsteen pair (see Figure 1A). Using the approximate shielding contours of Giessner-Prettre and Pullman (1970), and the projection of the helix down the axis given by Arnott and Bond (1973), we calculate similar extents of shielding from ring currents of the A and U nearest neighbors of the Watson-Crick ring proton (+0.88 ppm) and the U and A nearest neighbors of the Hoogsteen ring proton (+0.94 ppm). Thus there is no obvious major shielding effect to distinguish these two protons. However, if we accept an intrinsic shift of 14.6 ± 0.2 ppm for the U N_3H resonance in an A·U Watson-Crick pair, the above should lead to a resonance at 13.7 ppm, in reasonable agreement with the position of the lower field resonance observed, 13.8 ppm from DSS. If we assign this to the Watson-Crick proton, the resonance upfield at 13.2 ppm is that of the U N_3H, Hoogsteen pair; the intrinsic position for a Hoogsteen pair would therefore be approximately 14.1 ppm from DSS.

We have made several attempts to verify this assignment experimentally, without notable success. In one case, we added the analogue of adenosine, tubercidin, to the mixture. This nucleoside contains a ring C_7, instead of the normal

[2] The subscript to the oligomer represents the average number of residues in the oligomer.

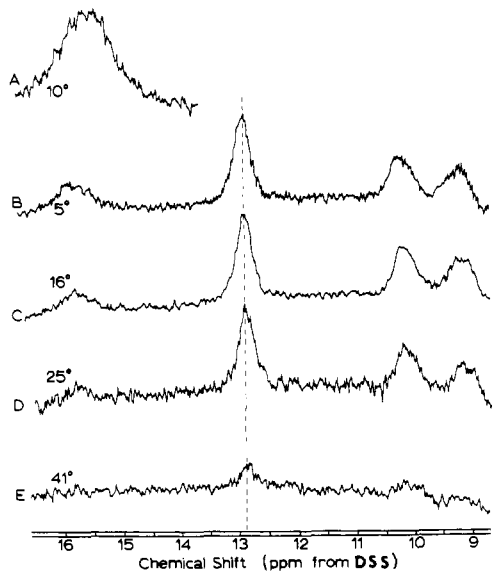

FIGURE 4: Low-field NMR spectrum of (A) oligo-C_{17} (154 mM in C) in 0.15 M NaCl, 0.01 M Tris-HCl, 5×10^{-4} M EDTA, pH 4.8; (B–E) mixture of GMP (23.5 mM) with oligo-C_{17} (154 mM in C) in 1.5 M NaCl, 0.01 M Tris-HCl, 5×10^{-4} M EDTA, pH 5.8, at indicated values of the temperature.

N_7, and cannot participate in Hoogsteen pairing. No relative change in intensity of the two resonances in Figure 3 resulted. Secondly, we prepared a mixture of oligo-U_{15} with N^6-methyladenosine, which is known to form double helical complexes with oligomers as well as polymers of U (Hoffman and Pörschke, 1973). No low-field resonances could be detected from this mixture, in accord with the rapid dissociation constant of 3×10^5 s^{-1} for this helix determined by relaxation methods (Hoffman and Pörschke, 1973).

3. Watson–Crick and Hoogsteen Base Pairs in the C·G·C^+ Complex. In the case of G and C, two different stable complexes have been identified in monomer–polymer mixtures (Howard et al., 1964): (1) a 1:1 helical complex between GMP and poly(C) stable at neutral pH; and (2) a 1:2 complex between GMP and two poly(C) strands favored at acid pH. This latter structure requires one protonated C base in each triple, permitting formation of a Hoogsteen pair between the protonated C and G as illustrated in Figure 1B. No low-field resonances corresponding to the 1:1 double helix at neutral pH were observed. However, the mixture of GMP with oligo-C_{17} at pH 5.8 reveals two low-field resonances of different line widths: one at 12.8 ppm, the other at 15.6 ppm downfield from DSS (Figure 4B–E). The 12.8 ppm resonance is clearly sharper than that at 15.6 ppm, and the two do not broaden out at the same temperature.

A crude ring current calculation based on the 12-fold helical structure analogous to the U·A·U helix which has been proposed for the C·G·C helix (Arnott and Bond, 1973) indicates a nearest neighbor contribution of +0.75 ppm at the position of the G N_1H of the Watson–Crick pair (see Figure 1B), and a +0.65 ppm shift at the position C N_3H proton of the Hoogsteen pair if it is assumed that the ring current in C^+ is approximately similar to that in C. Thus the predicted resonance for a Watson–Crick pair in this structure is about 13 ppm downfield from DSS, in fair agreement with the observed peak at 12.8 ppm. It seems reasonable to assign the 15.6 ppm resonance to the protonated G–C^+ Hoogsteen pair, the estimated intrinsic position of

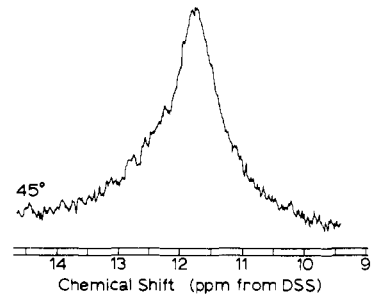

FIGURE 5: Low-field NMR spectrum of an alkaline hydrolysate of poly(I) (186 mM in I) in 1.5 M NaCl, 0.01 M Tris-HCl, 5×10^{-4} M EDTA, pH 6.7, at 45 °C.

which would be roughly 16.7 ppm from DSS. The difference in line widths of the two resonances is discussed below.

4. The C–C^+ Base Pair in Acid Oligo-C. Omission of the GMP from the oligo-C_{15} solution at pH 4 reveals a single broad low-field resonance at 15.5 ppm (Figure 4A), similar in both position and line width to the 15.6 ppm peak in C·G·C^+. Both adsorbance and CD measurements on oligo-C at this pH (Brahms et al., 1967) are consistent with helix formation, the presumptive structure of which is similar to the acid poly-C helix determined by Langridge and Rich (1963), shown in Figure 1C. This result thus tends to support assignment of the low-field resonance to the C^+ N_3H proton of the Hoogsteen pair in C·G·C^+.

5. The I_4 Base Quadruple in Oligo-I. At high salt concentration, poly(I) forms a helical complex (Davies and Rich, 1958), the structure of which is either a three- or four-stranded helix. The similarity of the fiber x-ray diffraction of poly(I) in high salt or in the presence of Mg^{2+} to that of the GMP gel or poly(G) has led to a reinterpretation of the original three-stranded model to one isomorphic with the four-stranded G helix (Zimmerman et al., 1975; Arnott et al., 1974); this is the structure shown in Figure 1D. Since this structure contains a nonstandard N—H··· O=C hydrogen bond involving a ring N_1-H proton and a carbonyl group, we investigated the low-field spectrum of an alkaline hydrolysate of poly(I) in H_2O. The unfractionated mixture, roughly of chain lengths 20 or so, but very heterogeneous, yielded the spectrum shown in Figure 5, with a single strong low-field resonance at 11.8 ppm from DSS. Thus even in cases where the helical structure is maintained (remarkably) by single interconnecting hydrogen bonds, the exchange of the N_1 proton is sufficiently slow to permit it to be observed by NMR.

6. Ring Protons in s^4U·A·s^4U Complex. The lowest downfield resonance (14.9 ppm from DSS) in the spectrum of tRNA's of *E. coli* containing the modified base 4-thiouracil (s^4U) at position 8 in the chain has been found to correspond to the s^4U N_3H proton of a tertiary pair A14 s^4U8 (Wong et al., 1975; Reid et al., 1975; Daniel and Cohn, 1975). We prepared oligo-s^4U of chain lengths 10–20 by amino–thiol exchange of a mixture of oligo-C in the presence of H_2S and pyridine for 3 weeks. The product exhibited the spectral properties of poly(s^4U) as was verified using a sample obtained from Y. Lapidot. A roughly equimolar mixture of this material with AMP in high salt was found to give resonances that broadened out below those of the corresponding U·A·U complex (Figure 3). We therefore added 15% (v/v) methanol (Patel and Tonelli, 1974) to prevent freezing and obtained the low-field spectra shown in Figure 6. Two peaks are observed, of roughly equal magnitude, one at 13.2 ppm, the other at 13.4 ppm. These broad-

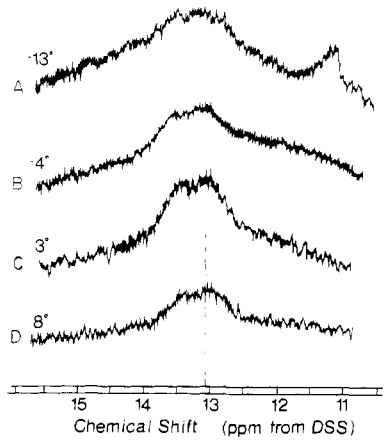

FIGURE 6: Low-field NMR spectrum of a mixture of AMP (100 mM) and oligo-s^4U_{15} (140 mM in s^4U) in 1.5 M NaCl, 0.01 M Tris-HCl, 5 $\times 10^{-4}$ M EDTA, pH 6.8.

en to similar extents without appreciable shifting, as T increases so that the exchange again appears to be opening-limited. The corresponding poly(s^4U)poly(A)·poly(s^4U) complex has been studied by Simuth et al. (1970), who report two distinct T_m values for this system, as well as a lack of stabilization by Mg^{2+} of the ordered form. Thus we have no way of knowing that the hydrogen-bonded structure present is analogous to U·A·U, although optical mixing experiments indicate a stable structure of 2:1 stoichiometry s^4U·A (Simuth et al., 1970). What is interesting in the spectra of Figure 6 is the lack of any appreciable resonances further downfield than those in U·A·U for instance. This result clearly does not imply that the intrinsic position of the s^4U·A Watson–Crick and/or Hoogsten base pairs is not downfield from those of the analogous U·A pairs. It is possible, for example, that the ring current shielding exerted by s^4U bases may be much stronger than by U, hence leading to the observed positions of the N_3H proton resonances slightly upfield from those in U·A·U. The similarity of the resonances in Figures 3 and 6 suggests that these two complexes may indeed have similar three-helical structures, although assignment of the downfield resonance to a Watson–Crick s^4U·A proton would be premature in view of the unknown shielding properties of s^4U and structural details of the s^4U·A·s^4U helix. The resonance at 11.2 ppm in panel A of Figure 6 is of presently unknown origin. It seemed possible that it represents the terminal resonances of the helix, by analogy to the situation in dA_3GCT_3 described above. However, our preliminary indication that it shifts *downfield* with increasing T below −4 °C makes this uncertain.

Discussion

These spectra show that resolvable resonances due to slow exchange of the ring protons—N_1H of guanine or hypoxanthine, N_3H of U, T or protonated C—involved in Watson–Crick as well as Hoogsteen and other nonclassical base pairings can be detected in aqueous solutions of synthetic oligonucleotides or mixtures of monomers with oligomers. The nonstandard associations we have investigated include a double helix, C·C^+, triple helices (U·A·U, C·G·C^+, and s^4U·A·s^4U) as well as the possible four-stranded structure, I_4. As in all the double helical structures so far reported, the onset of "broadening out" of the low-field ring proton resonances in hydrogen-bonded nucleic acids appears to occur at temperatures below the actual dissociation temperature (T_m) of the structure present, determined by uv or ir spectroscopy for example.

The exchange of protons involved in hydrogen bonds has been interpreted in terms of a kinetic scheme applied first to hydrogen–tritium exchange by Linderstrom-Leng (see Teitelbaum and Englander, 1975a,b, for references). In this scheme, three states are presumed available to protons: (1) within a hydrogen-bonded structure, designated a closed state; (2) associated with a transiently parted hydrogen-bonded structure so as to be available for exchange, designated an open state; and (3) transferred via the chemical exchange pathway(s) to the acceptor molecules, designated the exchanged state. Thus, the simplest kinetic formulation leads to a three-state process with three associated rate constants:

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftarrows}} \text{open} \xrightarrow{k_{ch}} \text{exchanged} \qquad (1)$$

the equilibrium constant for the conformational opening process being $K_{eq} = k_{op}/k_{cl}$.

The characteristics of this exchange process are by now well-known (Teitelbaum and Englander, 1975a; Crothers et al., 1974) so that it suffices for this discussion to observe that there exist two simple extreme possibilities for the overall exchange rate of eq 1. If the chemical process is fast, and $k_{cl} \ll k_{ch}$, the exchange follows the open limited pathway:

$$k_{ex} = k_{op} \qquad (2a)$$

on the other hand, if $k_{cl} \gg k_{ch}$, the exchange proceeds by the preequilibrium pathway:

$$k_{ex} = K_{eq}k_{ch} \qquad (2b)$$

Exchange of base protons in nucleic acids is subject to general acid or base catalysis, and for ring sites the maximum value of k_{ch} can be estimated from Eigen's expression:

$$k_{ch} \simeq 10^{10}(C)\frac{10^{\Delta pK}}{1 + 10^{\Delta pK}} \qquad (3)$$

with $\Delta pK = pK(\text{acceptor}) - pK(\text{donor})$.

Since k_{ch} estimated by eq 3 is 10^5 s^{-1} in the presence of 10 mM Tris buffer at neutral pH for either N_1H of G or N_3H of U or T, the exchange of the G·C ring protons (right-hand peak of Figure 2) is open-limited, and the opening rate is likely to be (Patel and Hilbers, 1975) the strand dissociation step (Reisner and Romer, 1973). Inspection of Figure 2 shows also that steps beyond strand association exert a major role in the exchange of the T N_3H resonances of dA_3 G·C T_3: (1) the position of the T N_3H resonance at 13.7 ppm shifts appreciably prior to broadening out near 10 °C; and (2) the ratio of the amplitudes of the internal of A·T to G·C resonances diminishes with increasing temperature (Figure 2A–E). Exchange of protons in base pairs involved in short-lived intermediate states that arise by opening and reclosing base pairs in a nucleated helix can lead to exchange approximating the preequilibrium pathway (eq 2b). As in the case of the dATGCAT helix (Hilbers and Patel, 1975), the spectra in Figure 2 can be reconciled quantitatively with a model in which the G N_1H resonances correspond to the open-limited case, and the internal T N_3H resonances to the preequilibrium case. There still remain quantitative discrepancies in accounting for the position and line width of the terminal A·T pairs in dA_3GCT_3, presumably due to some aggregation process involving the ends of the helix.

For the case of monomer–oligomer complexes, it is reasonable to equate the conformational process to dissociation of monomer from helix to free monomer in solution since release of monomers from double or triple helices can occur at any site of the chain, although still preferentially at the ends rather than from the interior (Hoffman and Pörschke, 1973). Given the condition of neutral pH and the presence of Tris buffer as catalyst, the dissociation constant k_{op} can be estimated from the increase in line widths in Figures 3, 4, and 6. In these complexes there is substantial contribution to T_2 from the dispersion of oligomer sizes present. For the U·A·U structure, line widths of the resonances assigned to the Watson–Crick and Hoogsteen U N_3-H increase in parallel as temperature rises. This suggests a common exchange mechanism for the two protons; in the case of the G N_1H and C^+N_3H peaks (Figure 4), this may not necessarily be so.

Comparison of the predicted and observed line widths for the C N_3H and G N_1H resonances provides a direct argument favoring the opening-limited mechanism for the C·G·C^+ complex. Using eq 3, we find that the anticipated proton transfer rates for the C proton exceed that for the G by a factor of 20. Clearly, the difference in line width between the 13 and 15.6 ppm resonances is far below this. Hence the preequilibrium dominated pathway (eq 2b) can be eliminated since the exchange rate in this pathway is directly proportional to k_{ch}. Any difference between the conformational opening equilibrium constants would serve to enhance this factor, not diminish it, because the Watson–Crick G·C pair is likely to be more stable than the Hoogsteen G·C^+ one, as seen by the fact that at neutral pH a stable double helix involving only the former can be detected (Howard et al., 1964). Apparently then exchange from C·G·C^+ is opening-limited. In no case have we been able to detect resonances from a double helical monomer–oligomer complex, presumably as a result of the correspondingly elevated k_{op} values (Hoffman and Pörschke, 1973).

Finally, the relevance of these results to structural studies of tRNA molecules in solution should be underscored. In each case so far examined of a complex involving base triples, we have detected low-field resonances of both the ring N protons. It is thus highly likely that the base pairs and triples involved in the tertiary structure of tRNA in solution should give rise to resonances in this low-field (10–16 ppm) region. A number of current estimates of the total number of slowly exchanging ring protons in tRNA in solution (Kearns and Shulman, 1974) repeatedly give values indicative of only the secondary structural base pairs. Most recently, careful measurements of the total number of slow protons in tRNA lead to substantially higher values, 26–27 (Reid et al., 1974; Daniel and Cohn, 1975), in accord with the excess number of slowly exchanging protons originally reported from earlier tritium–hydrogen exchange measurements (Englander et al., 1972; Goldstein et al., 1972). It should be emphasized that the base triples postulated in the tertiary structure of tRNA involve association of two purines with one pyrimidine, in contrast to the synthetic triple helices of two pyrimidines and one purine strand or component. Since the pairing within the two types of complex are similar (Hoogsteen, Watson–Crick, or their reverses), there is every reason to anticipate low-field resonances from these structures in view of our results.

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References

Arnott, S., and Bond, P. J. (1973), *Nature (London), New Biol. 244,* 99–101.

Arnott, S., Chaydras, R., and Marttila, C. M. (1974), *Biochem. J. 141*(2) 537–543.

Bock, R. M. (1967), *Methods Enzymol. 12*(A), 218–221.

Brahms, J., Maurizot, J. C., and Michelson, A. M. (1967), *J. Mol. Biol. 25,* 465–480.

Crothers, D. M., Cole, P. E., Hilbers, C. W., and Shulman, R. G. (1974), *J. Mol. Biol. 87*(1), 63–88.

Crothers, D. M., Hilbers, C. W., and Shulman, R. G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2899–2901.

Damle, V. N. (1970), *Biopolymers 9,* 353–272.

Daniel, W., and Cohn, M. (1975), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Davies, D. R., and Rich, A. (1958), *Nature (London) 182,* 955.

Egan, B. Z., and Kelmers, A. D. (1974), *Methods Enzymol. 12*(E), 469–472.

Englander, J. J., Kallenbach, N. R., and Englander, S. W. (1972), *J. Mol. Biol. 63,* 153–169.

Favre, A., and Fourrey, J. L. (1974), *Biochem. Biophys. Res. Commun. 58,* 507–515.

Giessner-Prettre, C., and Pullman, B. (1970), *J. Theor. Biol. 27,* 87–95.

Goldstein, R. N., Stefanovic, S., and Kallenbach, N. R. (1972), *J. Mol. Biol. 69,* 217–236.

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

Hoffman, G. W., and Pörschke, D. (1973), *Biopolymers 12,* 1611–1623, 1625–1638.

Hoogsteen, K. (1959), *Acta. Crystallogr. 12,* 822–823.

Howard, F. B., Frazier, J., Lipsett, M. N., and Miles, H. T. (1964), *Biochem. Biophys. Res. Commun. 17,* 93–102.

Howard, F. B., Frazier, J., Singer, M. F., and Miles, H. T. (1966), *J. Mol. Biol.* 415–439.

Kearns, D. R., and Shulman, R. G. (1974), *Acc. Chem. Res. 7,* 33–39.

Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman, J. L., Wang, A. H. J., Seeman, N. C., and Rich, A. (1974), *Science 185,* 435–440.

Langridge, R., and Rich, A. (1963), *Nature (London) 198,* 725–728.

Magee, W. S., Jr., Gibbs, J. H., and Zimm, B. H. (1963), *Biopolymers 1,* 133–143.

Miles, H. T., and Frazier, J. (1964), *Biochem. Biophys. Res. Commun. 14,* 21–28.

Patel, D. J., and Hilbers, C. W. (1975), *Biochemistry 14,* 2651–2656.

Patel, D. J., and Tonelli, A. E. (1974), *Biopolymers 13,* 1943–1964.

Reid, B. R., Ribeiro, N. S., Gould, G., Robillard, G., Hilbers, C. W., and Shulman, R. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2049–2053.

Riesner, D., and Romer, R. (1973), *Phys.-Chem. Prop.*

Nucleic Acids 2, 237-318.
Robertus, J. D., Ladner, J. E., Finch, J. J., Rhodes, D., Brown, R. S., Clark, B. F., and Klug, A. (1974), *Nucleic Acid Res. 1,* 927-932.
Scruggs, R. L., and Ross, P. D. (1970), *J. Mol. Biol. 47,* 29-40.
Shulman, R. G., Hilbers, C. W., Kearns, D. R., Reid, B. R., and Wong, Y. P. (1973), *J. Mol. Biol. 78,* 57-69.
Simuth, J., Scheit, K. H., and Gottschalk (1970), *Biochem. Biophys. Acta 204,* 317-380.
Stevens, C. L., and Felsenfeld, G. (1964), *Biopolymers 2,* 293-314.
Teitelbaum, H., and Englander, S. W. (1975a), *J. Mol. Biol. 92,* 55-78.
Teitelbaum, H., and Englander, S. W. (1975b), *J. Mol. Biol. 92,* 79-92.
Ueda, T., Imazawa, M., Mivra, K., Iwata, R., and Odajima, K. (1971), *Tetrahedron Lett. 27,* 2507-2510.
Wong, K. L., Bolton, P. H., and Kearns, D. R. (1975), *Biochim. Biophys. Acta 383,* 446-451.
Zimmerman, S. B., Cohen, G. H., and Davies, D. R. (1975), *J. Mol. Biol. 92,* 181-192.

Hydrogen-Bonded Complexes of the Ribodinucleoside Monophosphates in Aqueous Solution. Proton Magnetic Resonance Studies†

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ABSTRACT: A proton magnetic resonance study of the chemical shifts of a series of ribodinucleoside monophosphates in neutral H_2O solution has been recorded in the 1-100 mM concentration range. The self-complementary dinucleoside monophosphates CpG and GpC and the complementary mixture GpU + ApC form intermolecular hydrogen-bonded complexes at low temperatures. The amino proton chemical shifts in the CpG and GpC spectra are consistent with the formation of a miniature double helical dimer in neutral aqueous solution at low temperatures (~2 °C). The complementary mixture of dinucleosides GpU + ApC formed much less stable complexes than either GpC or CpG, while UpA did not show any indication of the formation of intermolecular hydrogen-bonded complexes. This result is consistent with the well-known observation that the stability of a double helix is proportional to the percent of G·C base pairs present.

The use of oligonucleotides of defined length and sequence as models for the corresponding regions in nucleic acids has provided valuable information on the properties of the nucleic acids (e.g., Jaskunas et al., 1968; Gennis and Cantor, 1970; Martin et al., 1971; Day et al., 1973; Uhlenbeck et al., 1973; Gralla and Crothers, 1973a,b; Borer et al., 1974, 1975; Ts'o, 1974a,b; and references therein; D'Albis et al., 1975; Hingerty et al., 1975). Optical spectroscopies have generally been used to investigate the properties of oligonucleotides. However, in the past few years nuclear magnetic resonance spectra of nucleic acids and proteins dissolved in an H_2O solvent have provided useful geometrical, kinetic, and thermodynamic information (e.g., Glickson et al., 1969; Kearns et al., 1971; Crothers et al., 1973; Patel and Tonelli, 1974; Arter et al., 1974; Krugh and Young, 1975; Young and Krugh, 1975; Borer et al., 1975). The NH resonance from G·C and A·T base pairs is a convenient probe for studying small double helical nucleic acids because this resonance is 5-9 ppm downfield from the large solvent resonance. However, the NH resonances broaden into the baseline at a temperature far below the T_m of the double helix and, thus, the extraction of thermodynamic parameters is not straightforward (e.g., Crothers et al., 1973; Borer et al., 1975). Mooberry and Krugh (1975) have shown that it is relatively easy to obtain pulsed Fourier transform proton magnetic resonance spectra in H_2O solutions. We have used these techniques to monitor the amino resonances of several deoxydinucleotides and have shown that complementary deoxydinucleotides interact by the formation of hydrogen-bonded dimers (Krugh and Young, 1975; Young and Krugh, 1975; see these papers for a more complete introduction to this area). An analysis of the concentration dependence of the amino resonances illustrated the effect of nucleotide sequence on the stability of the miniature double helices formed by two complementary deoxydinucleotides. In this paper we will investigate the interaction of several of the *ribo*dinucleoside monophosphates in neutral aqueous solution and show that, at temperatures near 0 °C, both CpG and GpC self-associate by the formation of intermolecular hydrogen-bonded complexes.

Experimental Methods

The dinucleoside monophosphates used in this study were purchased from either Sigma Chemical Co., or P-L. Biochemicals, Inc., and were used without further purification. Aqueous solutions of the ribodinucleoside monophosphates were initially made as concentrated as possible within the limits of solubility (or availability) of the compounds. Approximately 5 μl of 10^{-2} M disodium ethylenediaminete-

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