Nuclear Overhauser Studies of CCGGAp, ACCGGp, and ACCGGUp†

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ABSTRACT: Nuclear Overhauser effect (NOE) measurements are reported for the nonexchangeable base and ribose 1′ proton resonances of CCGGAp, ACCGGp, and ACCGGUp. The results permit assignment of these resonances to particular nucleotides in the sequences. The NOE data rule out conformations containing syn glycosidic linkages and conformations in which the purine 8 and pyrimidine 6 protons are closer to the 3′ nearest-neighbor ribose 1′ proton than to the 5′ nearest-neighbor ribose 1′ proton.

In the preceding paper (Petersheim & Turner, 1983a), we reported optical melting studies of the single-strand to double-helix transitions of CCGG, CCGGp, CCGGAp, CCGGUp, and ACCGGUp. Nuclear magnetic resonance (NMR)[1] can provide more detailed information on the structures of these molecules and their conformational changes (Petersheim & Turner, 1983b; Cross & Crothers, 1971; Kearns et al., 1971; Arter et al., 1974; Borer et al., 1975). To take advantage of this potential, it is necessary to assign the proton resonances of the oligomers. It has recently been shown that the nuclear Overhauser effect (NOE) is a powerful tool for assigning resonances in tRNA (Tropp & Redfield, 1981; Roy & Redfield, 1981; Hare & Reid, 1982). In this paper, we report use of NOE to assign resonances for the nonexchangeable base and ribose 1′ proton resonances of CCGGAp, ACCGGp, and ACCGGUp. In the following paper, this information is used to assign the resonances of CCGG, CCGGp, and CCGGUp and to interpret NMR melting curves (Petersheim & Turner, 1983b). The results indicate NOE provides an alternative to incremental analysis (Borer et al., 1975) and empirical shift parameters (Hader et al., 1982) for assignment of resonances.

The strong dependence of NOE on interproton distances can also provide a good measure of molecular structure in solution (Noggle & Schirmer, 1971; Cushley et al., 1972; Schirmer et al., 1972; Son et al., 1972; Tropp & Redfield, 1981; Wüthrich et al., 1978; Poulson et al., 1980). Interpretation of our results in terms of interproton distances is complicated by spin diffusion. Nevertheless, qualitative comparisons are made with NOE's expected from an A-RNA double helix.

Experimental Procedures

Samples. The oligomers were enzymatically synthesized as discussed elsewhere (Petersheim, 1982; Petersheim & Turner, 1983a). All samples were prepared by dissolving the oligomer in 1.0 M NaCl, 10 mM sodium cacodylate, 1 mM EDTA, and 1–10 mM TSP (internal reference), pH 7.0 in H_2O, and lyophilizing. The material was then treated with two cycles of dissolving in 99.8% D_2O (Bio-Rad) and lyophilizing. The dry sample was then flushed with dry nitrogen from a liquid nitrogen source and dissolved in 99.8% D_2O prepurged with dry nitrogen. The dissolved sample was kept under a nitrogen purge and transferred by syringe to a purged 5-mm NMR tube (Noe 527, Wilmad) equipped with a septum. Concentrations were determined from the absorbance at 280 nm, 70 °C (80 °C for ACCGGUp) (Petersheim & Turner, 1983a).

Instrumentation. Spectra were acquired on a Bruker WH400-MHz spectrometer with an Aspect 2000 computer. The temperature of the sample was regulated with a Bruker VT-1000 variable temperature unit. Temperatures were measured from the chemical shift difference between the resonances for neat methanol [cf. Petersheim & Turner (1983b)].

Spectra. Spectra were collected with quadrature detection, 16K words, and 4-kHz spectral width. Equilibrium spectra were obtained with a 2.48-s repetition rate and 100–1000 FID's, depending on the oligomer concentration. An NOE spectrum is essentially the difference between the equilibrium spectrum and a spectrum acquired after saturation of a selected resonance. Saturation of resonances in the NOE experiments was performed with the low-power decoupler source. A saturation time of 1 s immediately before acquisition was used; the repetition time was 3.48 s. The NOE spectra were collected as difference FID's in which the decoupler offset was placed on-resonance for half the scans and off-resonance by at least 200 Hz for the other half. The on-resonance FID's were negated and the off-resonance FID's added directly. Cycling between on- and off-resonance saturation was performed every 100 or fewer scans to reduce artifacts from temperature drift. The total number of scans was between 800 and 2000, depending on oligomer concentration. Measurements of T_1 were made by the inversion–recovery method.

Results

Resonance Assignments. Low-temperature spectra for the nonexchangeable base and ribose 1′ protons of CCGGAp, ACCGGp, and ACCGGUp in D_2O are presented in Figure 1. The other ribose resonances are highly overlapped with each other and HDO and are not included in this study.

As is generally the case (Jardetzky & Jardetzky, 1960; Borer et al., 1975), the region from 8.6 to 7.0 ppm contains resonances for A2, A8, G8, U6, and C6 protons. Resonances for U5, C5, and ribose 1′ protons occur between 6.2 and 5.0 ppm. There are several general rules for distinguishing the various resonances in each of these regions: (1) C6, C5, U6, and U5 occur as doublets (cytosine, J_{65} = 7.6 Hz; uracil, J_{65}

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; FID, free induction decay; ppm, parts per million; *J*, coupling constant; EDTA, ethylenediaminetetraacetic acid; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate.

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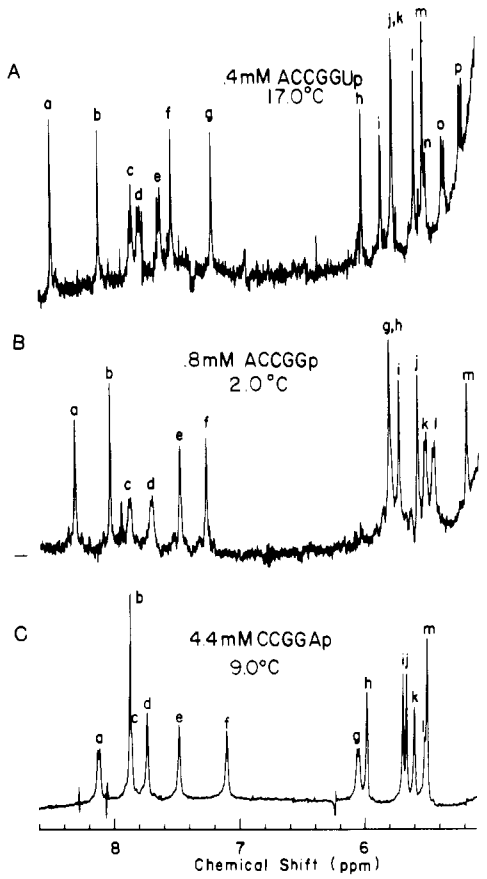

FIGURE 1: 400-MHz spectra of the nonexchangeable base and 1′ protons of CCGGAp, ACCGGp, and ACCGGUp. Resonance assignments are in Tables I–III. (A) 0.4 mM ACCGGUp at 17 °C (1200 FID's). (B) 0.8 mM ACCGGp at 2 °C (424 FID's). (C) 4 mM CCGGAp at 9 °C (200 FID's).

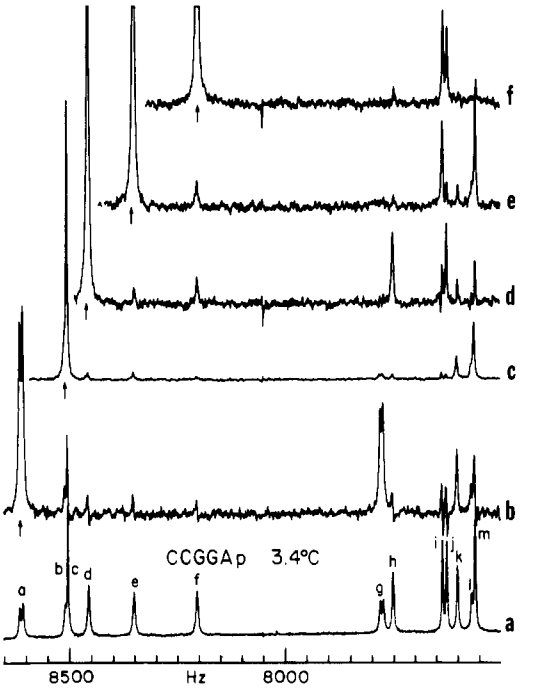

FIGURE 2: NOE difference spectra for 4.4 mM CCGGAp (1.0 M NaCl, 10 mM cacodylate, and 1 mM EDTA, 99.8% D_2O) at 3.4 °C. All observed NOE's are negative. At the bottom is the full spectrum from ~8.5 to ~5.5 ppm. The arrows indicate the on-resonance decoupler frequency; the off-resonance decoupler frequency was 8050 Hz relative to the 400-MHz carrier. The TSP resonance occurs at approximately 5335 Hz; in the difference spectra the TSP resonance was unreliable. In ascending order in the figure, the saturated resonances correspond to 1C6, 2C6/5A2, 5A8, 3G8, and 4G8. Each of the difference spectra represents 1000 FID's (500 on-resonance and 500 off-resonance); the full spectrum was obtained with 200 FID's. The noise in the NOE spectra was reduced by exponential multiplication of the FID resulting in a line broadening of 2 Hz; the full spectrum was broadened by 0.3 Hz. Complete NOE spectra are available as supplementary material and in Petersheim (1982).

= 8.1 Hz; Borer et al., 1975). (2) G8 protons exchange for deuterons much more rapidly than A8 protons at elevated temperatures (Pardi et al., 1981). (3) The spin–lattice relaxation time for A2 protons is approximately twice that of A8 and G8 protons (Pardi et al., 1981). (4) The 1′ protons can be distinguished from pyrimidine 5 protons by their temperature-dependent coupling to the 2′ protons. These rules were used to determine the proton types corresponding to each of the resonances in Figure 1.

In each of these oligomers there are two inequivalent cytosine and guanine residues and a ribose 1′ proton for each nucleoside residue. The resonances for these base and 1′ protons were assigned to particular residues on the basis of observed NOE's. A first-order NOE can be observed between two protons if they are separated by 4 Å or less, although there are several other considerations (Noggle & Schirmer, 1971; Bothner-By, 1979; Tropp & Redfield, 1981). In all possible conformations, the purine 8 and pyrimidine 6 protons are less than 4 Å from the 1′ proton of the associated ribose (Petersheim, 1982; Patel et al., 1982), and an NOE is expected.

Figure 2 presents equilibrium and NOE spectra for 4.4 mM CCGGAp at 3 °C. For all oligomers, all observed NOE's are negative. Since NOE spectra consist of small differences between spectra taken with and without presaturation, they are subject to artifacts. For example, in spectrum b of Figure 2, the derivative-like resonances probably result from chemical shift changes due to temperature fluctuations between the saturation and equilibrium experiments. Spectrum c apparently incurred a subtraction error as indicated by the residual peaks for all the resonances. Because of the artifacts, peaks were only considered due to NOE if they were greater than obvious artifacts. In practice, this meant that peaks with 5% or more of the area of the saturated resonance were considered due to NOE. Some resonances such as those at 8200 and 8350 Hz in spectrum d of Figure 2 were recorded as marginal but apparently real.

Table I: Observed NOE Resonances for CCGGAp[a]

peak designation	saturated resonance (ppm from TSP)	CCGGAp 12345 type	obsd NOE resonances[b-d]	assignment
a	8.1814	C6	b/c, g, k	1C6
b	7.9294	A2	a, (d)*, e, i, k, l/m	5A2
c	7.9221	C6	a, (d)*, e, i, k, l/m	2C6
d	7.7916	A8	b*/c*, f, h, j, (l/m)	5A8
e	7.5377	G8	b/c, g, i, l/m	3G9
f	7.1594	G8	(a), d, e, i, j	4G8
g	6.1100	C5		1C5
h	6.0404	1′	a, b, d, g*	5A1′
i	5.7512	1′		3G1′
j	5.72191	1′		4G1′
k	5.6585	1′		1C1′
l	5.5670	C5		2C5
m	5.5572	1′		2C1′

[a] 1.1 mM per strand, 7.1 °C, 1.0 M NaCl, 10 mM cacodylate, pH 7.0, and 1 mM EDTA, 99.9% D_2O. [b] Resonances b and c overlap, as do l and m. [c] Parentheses indicate small NOE peaks that may be artifacts. [d] Asterisks indicate peaks that may be due to tailing in the frequency distribution of the saturation power.

Table II: Observed NOE Resonances for ACCGGp[a]

peak designation	saturated resonance (ppm from TSP)	ACCGGp 1 2 3 4 5 type	obsd NOE resonances[b]	assignment
a	8.4017	A8	g, l	1A8
b	8.0527	A2	m	1A2
c	7.8880	C6	b*, e, j, k, m	3C6
d	7.7379	C6	e, g, l, m	2C6
e	7.5097	G8	f, i, j	4G8
f	7.3303	G8	e, h, i	5G8
g	6.0124	1′		1A1′
h	5.8647	1′		5G1′
i	5.7463	1′		4G1′
j	5.5840	1′		3C1′
k	5.5438	C5		3C5
l	5.4474	C5		2C5
m	5.2558	1′		2C1′

[a] 0.8 mM per strand, 7.3 °C, 1.0 M NaCl, 10 mM cacodylate, pH 7.0, and 1 mM EDTA, 99.9% D_2O. [b] Asterisk indicates a peak that may be due to tailing in the frequency distribution of the saturation pulse.

Table III: Observed NOE Resonances for ACCGGUp[a]

peak designation	saturated resonance (ppm from TSP)	ACCGGUp 1 2 3 4 5 6 type	obsd NOE resonances[b,c]	assignment[b]
a	8.5005	A8	(h), (o)	1A8
b	8.0881	A2	none	1A2
c	7.8605	C6	(f), l, (m), n	3C6
d	7.7952	C6	h, m, o	2C6
e	7.6372	U6	i, p	6U6
f	7.5377	G8	j/k, l	4G8
g	7.2168	G8	e, j/k	5G8
h	6.0050	1′		1A1′
i	5.8586	1′		6U1′
j	5.7805	1′		(G1′)
k	5.7744	1′		(G1′)
l	5.5975	1′		3C1′
m	5.5267	1′		2C1′
n	5.5029	C5		3C5
o	5.3382	C5		2C5
p	5.2240	U5		6U5

[a] 0.4 mM per strand, 6.6 °C, 1.0 M NaCl, 10 mM cacodylate, pH 7.0, and 1 mM EDTA, 99.9% D_2O. [b] Parentheses indicate peaks barely above noise. [c] j and k are indistinguishable in the NOE spectra because of noise. [d] j and k could not be unambiguously assigned.

A summary of the NOE's observed for CCGGAp, ACCGGp, and ACCGGUp is presented in Tables I–III. As is evident in Figure 2 and Tables I–III, saturation of either purine 8 or pyrimidine 6 protons generally yielded NOE's to two 1′ protons. It is reasonable to assume that one of the resonances corresponds to the 1′ proton of the ribose associated with the base. For a regular double-helical structure, the second 1′ resonance likely corresponds to a nearest-neighbor residue. Therefore, the resonance assignments were made with the assumption that NOE's to two 1′ protons corresponded to the associated and a nearest-neighbor ribose. There was sufficient information to obviate the assumption that a single NOE to a 1′ proton was necessarily to the associated ribose. In Figures 3–5, the observed NOE's are arranged to maximize the number of adjacent NOE's. The row labels correspond to the saturated resonance, and the column labels indicate the nucleoside residue for which an NOE is observed, both ordered according to sequence. The matrix elements indicate the

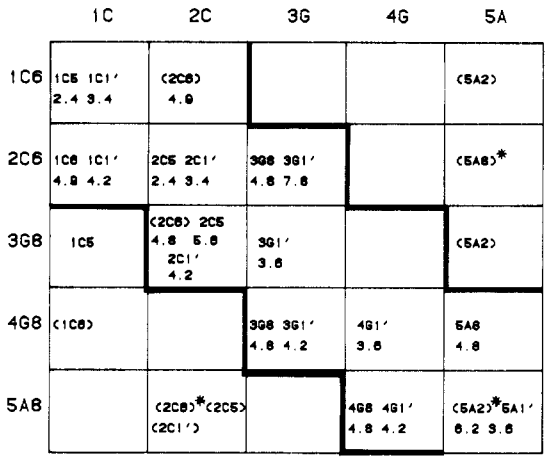

FIGURE 3: NOE results for 1.1 mM CCGGAp. The columns and rows are ordered by sequence. Row labels indicated the saturated resonance. Column labels indicate the nucleoside residue for which an NOE was observed. The matrix elements are the identities of the NOE resonances. The enclosed region about the diagonal delineates nearest neighbor relations. Parentheses indicate an NOE that may be an artifact. An asterisk indicates an NOE that may be due to tailing in the frequency distribution of the saturation power. The numbers beneath the NOE identities are A-RNA distances to the saturated proton (Petersheim, 1982).

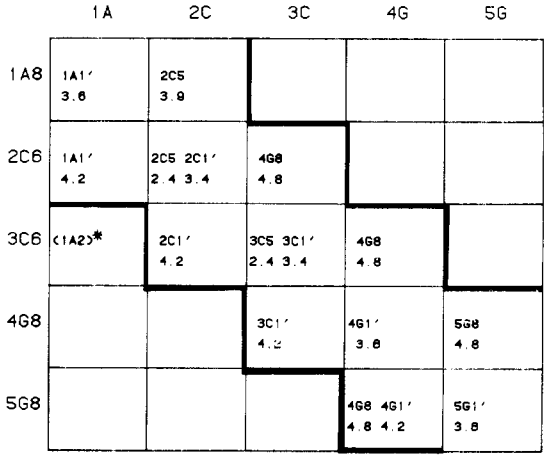

FIGURE 4: NOE results for 0.8 mM ACCGGp. See Figure 3 caption for details.

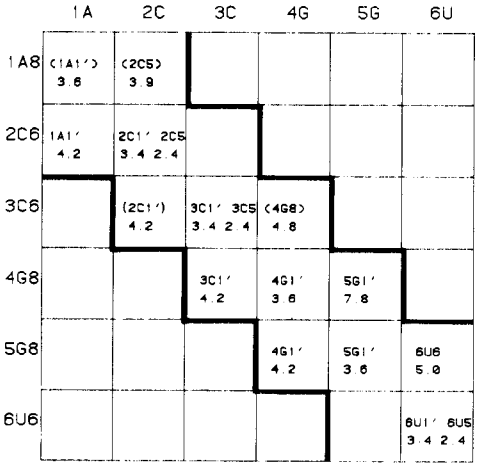

FIGURE 5: NOE results for 0.4 mM ACCGGUp. See Figure 3 caption for details.

proton for which an NOE was observed, and the interproton distances calculated for A-RNA structure are listed. The enclosed region about the diagonal delineates nearest-neighbor

Table IV: Comparison of NOE and T_1 Data for CCGGAp, ACCGGp, and ACCGGUp

		$\alpha\beta\gamma\delta$ CCGGAp			$\alpha\beta\gamma\delta$ ACCGGp			$\alpha\beta\gamma\delta$ ACCGGUp		A form
saturate (i)	observe (j)	% NOE_{ij}[a]	T_1 (s)	r_{ij}[c] (Å)	% NOE_{ij}[a]	T_1 (s)	r_{ij}[b,c] (Å)	% NOE_{ij}[a]	r_{ij}[b,c] (Å)	r_{ij} (Å)
A8	A1′	14	2.9	3.1	8	2.5	3.2	~6	3.4	3.6
γG8	γG1′	15	2.9	3.0	13	2.2	3.0	~5	3.5	3.6
δG8	δG1′	15	2.9	3.0	12	2.7	2.9	~8	3.1	3.6

[a] NOE_{ij} = area of j/area of i. [b] r_{ij} calculated from T_{ij} data for ACCGGp assuming τ_c = 2.5 ns. [c] Calculated distances are lower limits due to spin diffusion.

relations. Parentheses indicate results that are possibly artifacts.

The results indicate only two possible cases of NOE's from a purine 8 or pyrimidine 6 proton to a 3′-ribose 1′ proton. In ACCGGUp, the two ribose 1′ resonances from guanosine residues overlap. Thus it is impossible to tell if saturation of 4G8 gives a larger NOE to the 5′ or 3′ nearest-neighbor ribose 1′ proton. In CCGGAp, saturation of 2C6 produces NOE's to both the 5′ and 3′ nearest-neighbor ribose 1′ protons. However, the NOE to the 5′-ribose 1′ proton is much larger (see Figure 2). Thus it appears the saturated base protons are, in general, closer to the 5′ rather than the 3′ nearest-neighbor ribose 1′ proton.

In Figure 3, there are several non-nearest-neighbor NOE's recorded for CCGGAp, and in Figure 4 there is one recorded for ACCGGp. All but one of these arise from insufficient resolution of the saturated species. The 5A2 and 2C6 resonances of CCGGAp completely overlap at this temperature (cf. Figure 1 and Table I). Consequently, NOE's are recorded for both 5A2 and 2C6 from saturation of 1C6 and 3G8 even though all the observed NOE in that region is probably 2C6. The 5A8 resonance is within 50 Hz of the 2C6/5A2 resonances. At 50 Hz from the central saturation frequency, partial saturation was observed that was 4% of the maximum (Petersheim, 1982). The recorded NOE's for 5A8 and 2C6, when the other was saturated, are of this order and are probably due to this partial saturation. Saturation of 5A8 led to 2C5 and 2C1′ NOE's as well. C6 and C5 protons are 2.4 Å apart and demonstrate very large NOE's; observation of 2C5 probably results from partial saturation of 2C6. 2C1′ overlaps 2C5 (Figure 1 and Table I) and was recorded whenever an NOE was observed in that region. It may be present as a result of the partial saturation of 2C6. Observation of 1C5 upon saturation of 3G8 is anomolous. However, it was only observed at 1.1 mM CCGGAp. It is not present for 4.4 mM CCGGAp (See Figure 2d), and is probably an artifact at 1.1 mM. In ACCGGp (Figure 4), the NOE for 1A2 was small enough to be due to partial saturation from saturation of 3C6 even though the two resonances are 65 Hz apart. The partial saturation at 65 Hz was found to be 2–3% of the maximum (Petersheim, 1982). Most of the experiments were limited to saturation of the downfield resonances because the ribose 1′ and pyrimidine 5 resonances are not as well resolved (Figure 1). Therefore, partial saturation of adjacent resonances was a problem in this region (Petersheim, 1982).

Structure from NOE. In general, measurable NOE's are expected between protons separated by 4 Å or less. With this rule, the NOE's expected for CCGGAp, ACCGGp, and ACCGGUp were predicted from standard A-RNA geometry (Arnott et al., 1972). All the predicted NOE's were observed (see Tables I–III) except for 1A2–2C1′ and 6U6–5G1′ couples in ACCGGUp. The absence of these NOE's may result from the poor signal-to-noise ratio for ACCGGUp. As shown in Figures 3–5, several NOE's were also observed for protons separated by more than 4 Å. These may indicate double-helical structures different from A RNA. However, it is more likely they result from spin diffusion. The ribose 2′, 3′, and 4′ protons can provide efficient spin-diffusion pathways between adjacent nucleotides. Large NOE's were observed in the upfield ribose region indicating this [Petersheim, 1982; also see supplementary material (see paragraph at end of paper regarding supplementary material)]. For all but one of the NOE couples separated by >4 Å, there is at least one proton in the A-RNA structure that is less than 4 Å from both the saturated and observed protons. The one exception is the 3G8–1C5 NOE in 1.1 mM CCGGAp, discussed above. This appears to be an artifact. Therefore, all the NOE results are qualitatively consistent with A-RNA structure except for the lack of 1A2–2C1′ and 6U6–5G1′ NOE's in ACCGGUp.

In principle, NOE data can be used to obtain interproton distances to a few tenths of an angstrom (Tropp & Redfield, 1981). When relaxation is dominated by dipolar processes and spin diffusion is negligible, the interproton distance, r_{ij}, is calculated from (Tropp & Redfield, 1981; Kalk & Berendsen, 1976)

$$r_{ij} = \alpha\left(\frac{T_{1j}[1 - \exp(-t/T_{1j})]}{NOE_{ij}}\right)^{1/6} \quad (1)$$

$$\alpha = \frac{\gamma^4\hbar^2}{10}\left[\frac{6\tau_c}{1 + (2\omega\tau_c)^2} - \tau_c\right] \quad (2)$$

NOE_{ij} is the change in the resonance area for proton j upon saturating resonance i (for calculations, we have actually defined NOE_{ij} as the ratio in the NOE difference spectrum of the area of peak j to the area of peak i), τ_c is the rotational correlation time for i and j, T_{ij} is the spin–lattice relaxation time of proton j under the conditions of the NOE experiment, t is the saturation period, ω is the proton Larmor frequency (2.513×10^9 rad s^{-1} for these experiments), and γ is the proton gyromagnetic ratio (26 752 rad s^{-1} G^{-1}).

In the experiments reported here, spin diffusion will contribute to NOE_{ij} so that eq 1 provides a lower limit for r_{ij}. An additional complication is the difficulty in measuring T_{ij}. To obtain qualitative distances, we used the "nonselective" T_1 for T_{1j}. This is the relaxation time when all spin populations are equally perturbed from equilibrium by presaturation. This is not strictly the T_{1j} operative during the NOE experiments. However, it is probably a reasonable approximation for the qualitative calculations presented here (Petersheim, 1982).

Calculations of r_{ij} also require knowledge of α. α was estimated with eq 2 assuming the 1C6 to 1C5 distance is 2.44 Å (Arnott et al., 1972). The resulting value of α for CCGGAp at 3 °C was 135 $Å^6$ s^{-1}, which corresponds to $\tau_c = 2.5 \pm 1$ ns. From the Stokes–Einstein equation $\tau_c = V\eta/(RT)$, 2.5 ns is the rotational time for a sphere of 11 Å radius. This appears to be reasonable for CCGGAp.

Table IV lists distances that could be calculated for all three oligomers. It was assumed that α is the same for all the

oligomers and that the T_1's for ACCGGUp are the same as for ACCGGp. While these distances are lower limits and must clearly be considered qualitative, it is likely the approximations are of equal validity for all three oligomers. Comparison of the distances in the three oligomers suggests they have similar conformations.

Discussion

In order to tap the structural information available from NMR, it is necessary to assign the resonances to particular nuclei. Nuclear Overhauser effects have previously been used to make resonance assignments in tRNA (Tropp & Redfield, 1981; Roy & Redfield, 1981; Hare & Reid, 1982). This paper applies the method to small oligomers, namely, CCGGAp, ACCGGp, and ACCGGUp. It is found that NOE's are observed between the nonexchangeable base protons (A8, G8, C6, U6) and at least one, but in general two, of the ribose 1′ protons. These represent the ribose 1′ protons associated with the base and with its 5′ nearest neighbor. This permits the unambiguous assignment of all the nonexchangeable base and ribose 1′ protons, with the exception of the two G1′ resonances in ACCGGUp. These resonances are resolved by only 0.006 ppm and could not be distinguished in the NOE spectrum. The NOE approach takes less time than incremental analysis (Borer et al., 1975) and is more direct than empirical shift parameters (Hader et al., 1982). Thus it should be useful for oligomer studies.

Nuclear Overhauser effects can also provide structural information (Cushley et al., 1972; Schirmer et al., 1972; Son et al., 1972; Tropp & Redfield, 1981; Patel et al., 1982). Spin diffusion and the difficulty in measuring the appropriate spin–lattice relaxation time limit interpretation of the NOE spectra presented here. However, the results rule out conformations containing syn glycosidic linkages since these have purine 8 to ribose 1′ distances shorter than the lower limits measured here (Patel et al., 1982). They also rule out conformations in which the purine 8 and pyrimidine 6 protons are closer to the 3′ nearest neighbor ribose 1′ proton than to the 5′ nearest neighbor ribose 1′ proton, since NOE's to the 5′ ribose 1′ proton are larger than to the 3′ ribose 1′ proton. Qualitatively, the NOE results are consistent with standard A- and B-form geometries.

The results also indicate it will be possible to use NOE data to obtain accurate interproton distances in solution. This will require using shorter saturation pulses to limit spin diffusion and measuring the NOE as a function of the saturation pulse length, t. Such experiments will provide a set of equations like eq 1 that can be solved simultaneously for the spin–lattice relaxation time, T_{ij}, and the distance, r_{ij}.

Acknowledgments

We thank Dr. Susan M. Freier for stimulating discussions and Barbara Burger for artistic and computational assistance.

Supplementary Material Available

Five figures showing complete NOE spectra used for Figure 2 (5 pages). Ordering information is given on any current masthead page.

Registry No. CCGGAp, 83831-16-7; ACCGGp, 83831-17-8; ACCGGUp, 83831-19-0.

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