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Chemically Induced Dynamic Nuclear Polarization Studies of Guanosine in Nucleotides, Dinucleotides, and Oligonucleotides[†]

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ABSTRACT: The nuclear magnetic resonance (NMR) technique of chemically induced dynamic nuclear polarization (CIDNP) has been used to study the reactions between photoexcited flavins and a wide variety of nucleotides, dinucleotides, and oligonucleotides. The greatest emphasis is placed on the purine nucleosides adenosine (A), inosine (I), and guanosine (G), particularly guanosine. The presence of G suppresses the CIDNP effect for A, although A by itself shows very strong CIDNP [Kaptein, R., Nicolay, K., & Dijkstra, K. (1979) *J. Chem. Soc., Chem. Commun.*, 1092-1094]. Very intense CIDNP signals are observed for the H8 proton in G-containing mononucleotides, but no nuclear polarization is detected for the sugar H1' proton. In contrast, both H8 and H1' protons exhibit CIDNP for G in a wide range of dinucleotides and higher oligonucleotides. Several possible mechanisms are analyzed to explain the H1' polarization, and it is concluded

that the sugar H1' proton probably obtains spin density through interaction with guanine nitrogen 3. The proximity of the H1' proton to N3 depends explicitly on the glycosidic torsion angle, χ . CIDNP studies of several model compounds in which χ is fixed are consistent with this suggestion. CIDNP for the self-complementary tetramer ApGpCpU was studied as a function of temperature. Strong CIDNP from G is only observed at temperatures above the double-strand melting temperature, suggesting that CIDNP is only detected in single-stranded regions, where the base is accessible to solvent. The use of brominated riboflavin as the photoreagent in place of riboflavin is shown to selectively invert the sign of A, I, and 1-methylguanosine polarization, providing a convenient method for distinguishing the NMR spectra of these residues in complex oligonucleotides.

The conformations of nucleic acids and the forces responsible for maintaining the structures of oligonucleotides provide a basis for understanding their biological function. Nuclear magnetic resonance (NMR)¹ and circular dichroism (CD) have been the primary methods used to determine the conformations of nucleotides, oligonucleotides, and nucleic acids in solution [Sarma (1980) and references cited therein; Davies, 1978a; Johnson, 1978]. Measurements of vicinal coupling constants can provide values (or ranges of values for species equilibrating between conformations) for the backbone torsion angles β , γ , δ , and ϵ but not for the glycosidic torsion angle

χ or for the phosphodiester bond angles α and ζ (see G in Figure 1 for notation). At low temperatures, the phosphodiester bond angles are assumed to be predominantly in the $\zeta^- \alpha^-$ conformation, on the basis of chemical shift calculations and consideration of molecular models [Sarma (1980) and references cited therein].

It is generally assumed that in neutral aqueous solution all naturally occurring nucleic acids exist as an equilibrium mixture of syn ($-90^\circ < \chi < 90^\circ$) and anti ($90^\circ < \chi < 270^\circ$) conformations, with a preference for the anti conformation (Lee et al., 1976). Indirect NMR parameters can be used to estimate χ , such as the chemical shifts of the base and ribose protons as a function of temperature, pH, and various substituents, changes in chemical shifts and relaxation times upon

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¹ Abbreviations: CD, circular dichroism; CIDNP, chemically induced dynamic nuclear polarization; 8BrF, 7-methyl-8-bromo-10-(1-D-ribityl)isoalloxazine; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance. Standard abbreviations are used for nucleic acids (Davies, 1978a; Nishimura, 1978). Nomenclature used for torsion angles in the nucleotides is from Davies (1978b); all references to angles from other sources have been converted to this nomenclature.

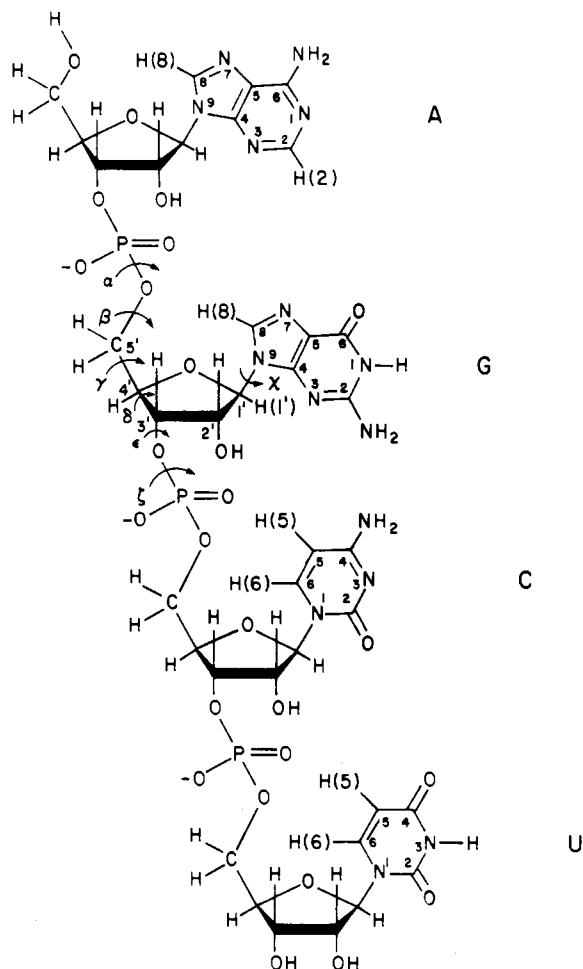


FIGURE 1: Structure and numbering system for ApGpCpU. The backbone and glycosidic torsion angles are illustrated for G.

metal binding, and ^{13}C - ^1H and long-range ^1H - ^1H coupling constants. In some cases, these values are in conflict with those derived from T_1 and nuclear Overhauser enhancement (NOE) measurements [Sarma (1980) and references cited therein; Davies, 1978]. In dinucleotides, χ has been estimated from proton chemical shift values as a function of pH, temperature, and composition by consideration of molecular models and by T_1 measurements [Sarma (1980) and references cited therein; Davies, 1978; Neuman et al., 1979; Chachaty et al., 1977, 1980; Tran-dinh et al., 1981].

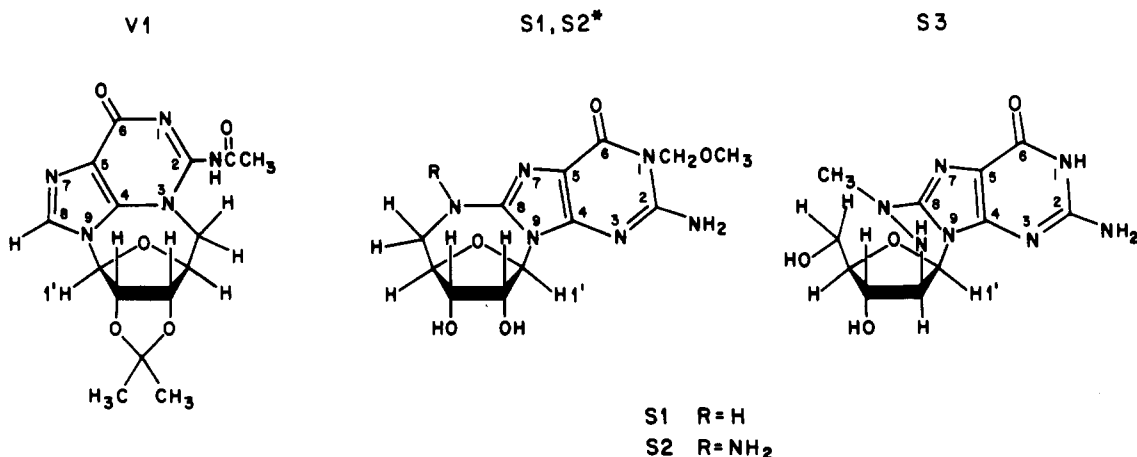


FIGURE 2: Structures and numbering systems of (V1) *N*²-acetyl-2',3'-O-isopropylidene-3,5'-cycloguanosine, (S1) 8,5'-imino-1-(methoxymethyl)-9-(5-deoxy-β-D-ribofuranosyl)guanine, (S2) 8,5'-(aminoimino)-1-(methoxymethyl)-9-(5-deoxy-2,3-O-isopropylidene-β-D-ribofuranosyl)guanine, and (S3) 8,2'-(*N*^α-methylhydrazino)-9-(2-deoxy-β-D-arabinofuranosyl)guanine. (*) The 2',3'-O-isopropylidene group of S2 is not shown in the figure.

In the present paper, we demonstrate that the NMR technique chemically induced dynamic nuclear polarization (CIDNP) can be used to study conformation and solvent accessibility in oligonucleotides and nucleic acids. Photo-CIDNP has been used extensively by Kaptein and his co-workers to study the surface accessibility of His, Tyr, and Trp residues in proteins to photoexcited flavin dyes (Kaptein, 1980). CIDNP is observed as greatly enhanced absorption or emission of the NMR resonances of the diamagnetic products of reactions in which radical pairs are intermediates. The observed nuclear polarization for a reversible reaction is the consequence of incomplete cancellation of the polarization in geminate recombination and escape pathways due to nuclear relaxation in the escaping radicals.

Kaptein has also shown that CIDNP is observed for Ada and Gua bases, nucleotides, and nucleosides, for the pyrimidines Thy, 5MeCyt, and 3MeCyt (Kaptein et al., 1979), and for the dimers d(ApA) and d(pApA) (Kaptein et al., 1981) following reaction with photoexcited riboflavin. Solutions of riboflavin and the oligomers d(pCpGpCpG) (Garssen et al., 1978), d(pCpGpCpGpCpG) (Hilbers et al., 1978), oligo[d-(AT)] (Buck et al., 1980), and poly(A) (Garssen et al., 1978), however, showed no CIDNP following laser irradiation. In the present work, we look at CIDNP in other nucleotides and oligonucleotides, with particular emphasis on the G polarization. In the following paper (McCord et al., 1984), these studies are extended to yeast tRNA^{Phe}.

Experimental Procedures

Nucleic acid monomers and dimers and ApGpU were purchased from Sigma, Aldrich, or P-L Biochemicals. ApGpCpU was a generous gift from Dr. P. Borer of Syracuse University and was also synthesized by enzymatic methods (Martin et al., 1971; Uhlenbeck et al., 1971). The structure and numbering system of ApGpCpU are shown in Figure 1 for reference. *N*²-Acetyl-2',3'-O-isopropylidene-3,5'-cycloguanosine (V1) was a generous gift from Dr. J. P. H. Verheyden of Syntex Corp., Palo Alto, CA. 8,5'-Imino-1-(methoxymethyl)-9-(5-deoxy-β-D-ribofuranosyl)guanine (S1), 8,5'-(aminoimino)-1-(methoxymethyl)-9-(5-deoxy-2,3-O-isopropylidene-β-D-ribofuranosyl)guanine (S2), and 8,2'-(*N*^α-methylhydrazino)-9-(2-deoxy-β-D-arabinofuranosyl)guanine (S3) were a generous gift of Professor Sasaki, Nagoya University, Japan. These compounds will be denoted V1, S1, S2, and S3, respectively; their chemical structures are illustrated in Figure 2. 7-Methyl-8-bromo-10-(1-D-ribityl)iso-

alloxazine (denoted 8BrF) was a generous gift from Dr. J. Lambooy at the University of Maryland.

Typically, samples were prepared by combining the nucleotide dissolved in D₂O and 0.5 mL of deuterated pH 7 Sorensen's phosphate buffer (usually 66 mM phosphate) saturated with riboflavin (0.4 mM), followed by lyophilization and dissolution in D₂O (100% D, Aldrich). The pH values of deuterated solutions were measured with a glass electrode and were not corrected for the isotope effect. The 8-proton of the purines was exchanged for deuterium by heating the samples in an 80 °C water bath in the sealed NMR tube for 3 h.

NMR data were obtained on the 360-MHz NMR spectrometer at the Stanford Magnetic Resonance Laboratory. The chemical shifts are referenced to internal 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) except the ApGpCpU samples, which were referenced to 3-(trimethylsilyl)propionate (TSP). Presaturation of the HDO resonance was used to suppress the signal from the residual water in the samples. The temperature was 25 ± 1 °C unless otherwise indicated. T_1 data were collected on the same samples used for the CIDNP experiments by a standard inversion-recovery pulse sequence; the T_1 values are accurate to ± 0.2 s. The photo-CIDNP apparatus and data collection system have been previously described (McCord et al., 1981). CIDNP spectra were obtained with a 90° radio-frequency pulse. Enhancement factors are defined as the difference between the integrated intensity of a polarized peak and its unenhanced dark intensity, divided by the latter. These values can be interpreted relative to each other, but not in absolute terms, as the absolute CIDNP intensity depends on sample geometry, dye concentration, etc. The T_m value of ApGpCpU was determined by measuring the temperature dependence of the absorbance at 260 nm on a Gilford Model 250 spectrophotometer (Nelson et al., 1981).

Results

Monomers. Solutions of riboflavin (0.2 mM) and the following molecules were studied: uracil (Ura), thymine (Thy), cytosine (Cyt), inosine (I), guanosine (G), uridine (U), adenosine (A), 3'-GMP, 3'-UMP, 3'-AMP, 3'-CMP, 3'-IMP, 3'-dTMP, 5'-GMP, 5'-CMP, 5'-AMP, 5'-UMP, 5'-dAMP, 5'-dTMP, 9-methylguanine (9MeGua), and 5-methylcytosine (5MeCyt). Solutions were either 33 or 66 mM phosphate, 2.5 mM in base, except for 9MeGua, which was a saturated solution (~ 1.5 mM), and were pH 7, except for 5'-CMP, 5'-AMP, 5'-UMP, and 5'-dAMP, which were pH 5. Samples of 5'-GMP were examined at both pH 5 and 7. I (2.5 mM) and G (2.5 mM) solutions containing various phosphate concentrations (0–750 mM) and 0.4 mM riboflavin at pH 7 were also examined, as well as solutions of G as a function of pH (pH 5–10, phosphate or borate buffer), and at pH 7 (66 mM phosphate buffer) as a function of temperature. The results are summarized in Table I.

The I(H8) and G(H8) enhancements decrease monotonically as the phosphate concentration increases; at 750 mM phosphate, the enhancement is about half that at zero phosphate concentration. The sign of the polarization does not change as the phosphate concentration is varied. The G polarization is strongest at pH 7–8 and decreases at both lower and higher pH. The polarization decreases as the temperature is increased; the enhancement at 55 °C is approximately half that at 20 °C. The polarization also decreases $\sim 15\%$ as the temperature is lowered to 5 °C. The sign and magnitude of the A(H8) and A(H2) polarization are highly variable, as previously discussed (Scheek et al., 1981). Polarization is only observed for U, T, and C derivatives if the 1-proton (or deu-

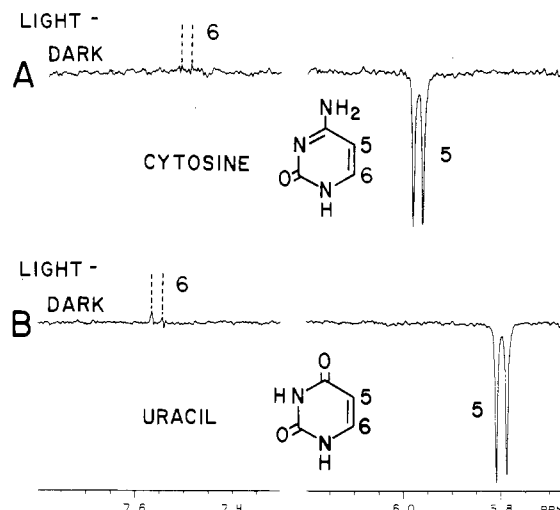


FIGURE 3: ¹H light minus dark difference spectra (360 MHz, one accumulation each) in pH 7 buffer containing 66 mM phosphate and 0.4 mM riboflavin at 25 °C. Dotted lines indicate resonances that are not spin polarized and therefore cancel in the difference spectrum. (A) Cytosine (2.5 mM); (B) uracil (2.5 mM).

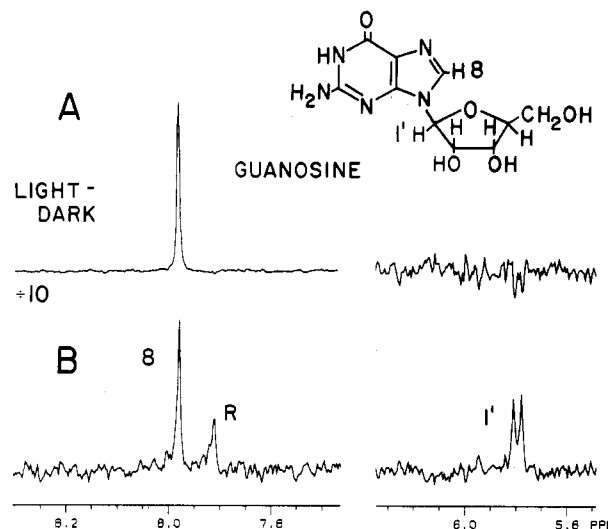


FIGURE 4: ¹H NMR spectra (360 MHz, one accumulation each) of guanosine (2.5 mM) in pH 7 buffer containing 66 mM phosphate and 0.4 mM riboflavin at 25 °C. The structure illustrates the anti conformation. (A) Light minus dark difference spectrum; (B) dark spectrum. R indicates riboflavin.

teron) is present. Thus, H5 shows negative polarization in Ura and Cyt (Figure 3), while the 5-methyl protons in 5MeCyt and Thy show positive polarization, and no polarization is observed for U, 3'-UMP, 3'-CMP, 5'-UMP, or 5'-dTMP.

The G(H8) resonance shows positive CIDNP in all cases (Figure 4). The measured G(H8) enhancement factors for most of the monomers are in the range of 4–8, while that of 9MeGua is 11.5. Polarization of this proton can easily be observed in solutions of G in a single scan at a concentration as low as 0.06 mM. Polarization of the 1'-sugar proton in G (Figure 4) and 5'-GMP or of the methyl protons of 9MeGua is extremely weak and barely detectable at 6 °C. No sugar polarization is observed in 9MeGua, 5'-GMP, or G when the H8 proton is either saturated with radio frequency (rf) during the light pulse or deuterated. The T_1 values for H8 and H1' in G are 1.8 and 2.4 s, while those for H8 and the methyl protons in 9MeGua are 5.2 and 1.3 s, respectively. In solutions containing both A and G, the relative amount of A polarization as compared to the G polarization varies depending on the A and G concentrations. When both nucleosides are 2.5 mM

Table I: Summary of Spin Polarization of Mononucleotides upon Reaction with Photoexcited Riboflavin^a

monomer	H2	H8	H5	H6	H1'	other
Ura			+	n		
U			n	n	n	
3'-UMP			n	n	n	
5'-UMP			n	n	n	
Thy			+Me	n		
3'-dTMP			n	n	n	
5'-dTMP			n	n	n	
Cyt			—	n		
3'-CMP			n	n	n	
5'-CMP			n	n	n	
5MeCyt			+Me	n		
A ^b	+w+	+/-			n	
3'-AMP ^b	+w+	+/-			n	
5'-AMP ^b	+w+	+/-			n	
5'-dAMP ^b	+w+	+/-			n	
G ^c		+			w+	
3'-GMP		+			n	
5'-GMP		+			w+	
9MeGua		+			wMe	
I ^d	—	—			n	
3'-IMP ^d	—	—			n	
m ¹ G		—			n	
m ² G		+			n	—Me ^e
D			n	n	n	
Gm		+			n	nMe
Ψ				w+	+	
m ⁷ G		n ^e			w	+Me
m ¹ I	n	n			n	
2-aminopurine		+		+		
8-methyl-3',5'-cycloadenosine	+	—Me			n	
purine	n	n		n		

^a Symbols used in the table are as follows: +, positive polarization; —, negative polarization; w, weak polarization; n, no observed polarization; Me, polarization of methyl protons. ^b The intensity and sign of the polarization are dependent on temperature, on buffer concentration, and on the presence of ionized phosphate groups in the nucleotide (Scheek et al., 1981). ^c Polarization is greatest at pH 7–8 and decreases when the pH is either lower or higher than this range. The polarization is temperature dependent at pH 7 (see text). ^d The polarization decreases as buffer phosphate concentration increases; however, no change in the sign of the polarization is observed. ^e The solubility of these molecules is very temperature dependent (see text). ^f This proton exchanges rapidly with ²H.

(0.4 mM riboflavin), the relative G(H8) to A(H8) polarization is 18 to 1, whereas, when both nucleosides are 0.1 mM (0.4 mM riboflavin), the relative G(H8) to A(H8) polarization is 0.9 to 1.

Solutions of the modified or rare bases 1-methylguanosine (m¹G), N²-dimethylguanosine (m²G), N²-methylguanosine (m²G), dihydrouridine (D), 2'-O-methylguanosine (Gm), pseudouridine (Ψ), 7-methylguanosine (m⁷G), 1-methylinosine (m¹I), 2-aminopurine, 8-(methylamino)adenine, and purine were studied. m¹G shows weak negative polarization of the G(H8) resonance (enhancement factor about -2). m²G is nearly insoluble and shows very weak negative polarization of the methyl protons at 25 °C. The solubility of this compound is very temperature dependent. At 40 °C and above, strong CIDNP is observed for m²G(H8) and for the methyl protons. At 40 °C, the ratio of the enhancements of the H8 to the methyl resonance is 1 to -2.5. Separately, m²G and G have almost the same enhancement factor for H8 when each is 0.3 mM in 0.25 mM riboflavin, pH 7 (66 mM phosphate) solution at 40 °C. In an equimolar mixture of these two compounds (1 mM each) under the same conditions, the 8-protons again showed almost identical enhancements. m²G is also increasingly soluble at higher temperatures. It, too,

shows positive m²G(H8) polarization and negative methyl proton polarization. Dihydrouridine shows no polarization, and pseudouridine shows weak positive polarization of H1'. Gm shows only positive H8 polarization. In m⁷G, H8 is not observed due to the fast exchange of this proton with deuterium. H1' shows weak positive polarization, and the 7-methyl protons show strong positive polarization. m¹A shows positive polarization of H8. m¹I and purine itself show no polarization, while 2-aminopurine shows positive polarization for H8 and H6. 8-(methylamino)adenosine shows positive polarization of H2 and negative polarization of the methyl resonance.

A series of synthetic purine mononucleoside derivatives with fixed angle χ were also studied in pH 7 solution, 66 mM phosphate buffer, 0.4 mM riboflavin (Figure 2). The cyclic derivative V1 gives strong positive polarization for H8 and weak negative polarization for H1', H5', H5'', and the methyl proton resonances of the acetyl group. The polarization of H1' disappears when H8 either is deuterated or saturated with rf during the light pulse. S3 shows positive polarization of the 8-(aminomethyl) protons and of H2' and weak negative polarization of H1'. The weak negative polarization of H1' was not affected by rf irradiation of H2' during the light pulse. S1 and S2 show very weak negative polarization of H1' and of the N1 methylene protons. It should be cautioned that weak negative polarization could well be an artifact in light minus dark spectra arising from line broadening and loss of signal intensity due to minor heating effects of the laser. S2 shows weak positive polarization of the 5'- and 5''-protons, while S1 shows weak negative polarization of these protons. Upon a raising of the pH to 9–10 by the addition of NaOD, S2 shows positive polarization of H1', H5', and H5''. This polarization disappears when the pH is subsequently lowered to 5. S1 also shows increasing positive polarization of H1', H5', and H5'' as the pH is increased to 10. S2 decomposes readily in aqueous solution to a compound that shows only weak positive polarization of the 5'- and 5''-protons at any pH. Deoxyribo-1-isobutylguanosine 5'-(cyanoethyl phosphate) shows negative polarization of H8 only. This reversal of sign is probably due to an increase in the magnitude of the *g* factor caused by the substituent at N1 (vide infra).

Solutions of 8BrF (about 0.3 mM) and either I, m¹I, G, m¹G, or A (2.5 mM) in pH 7, 66 mM phosphate buffer were examined. The enhancements for H8 are as follows: G, 18; m¹G, 8; I, 2; m¹I, 0; A, 5; A (no phosphate), 6. By contrast, the enhancements with riboflavin as the dye are as follows: G, 9; m¹G, -3; I, -8; m¹I, 0; A, -7. As the extinction coefficient, intersystem crossing yield, and reactivities of the two flavins may well be different, the absolute magnitudes of these enhancements are not particularly meaningful; however, it should be noticed that in three cases, the *sign* of the polarization changes.

Dimers and Trimer. Solutions of the following were studied: CpG, GpA, ApG, GpC, GpU, UpG, GpG, d(CpG), d(TpG), d(pCpG), d(pGpG), d(pGpA), CpGd8, ApGpU, IpI, ApC, CpA, UpA, ApA, d(pApT), d(CpA), d(ApT), 2',5'-GpA, and 2',5'-ApG. Solutions of 5'-phosphate-containing molecules and d(CpG) were pH 5; all other solutions were pH 7. Solutions were 33 or 66 mM phosphate buffer; the phosphate concentration did not affect the relative G(H8) to G(H1') polarization. Nucleic acid concentrations were 1–2.5 mM. The results are summarized in Table II.

A(H8) and A(H2) show polarization *unless* the molecule contains G; in molecules containing both G and A, no polarization is observed for the A protons. This was true for ApG

Table II: Summary of Spin Polarization of Dinucleotides and Oligonucleotides upon Reaction with Photoexcited Riboflavin^a

	A(H2)	A(H8)	G(H8)	G(H1')
CpG			+	+
GpA	n	n	+	+
ApG	n	n	+	+
GpC			+	+
GpU			+	+
UpG			+	+
GpG ^b			+	n
d(CpG)			+	+
d(TpG)			+	+
d(pCpG)			+	+
d(pGpG) ^b			+	n
d(pGpA)	n	n	+	+
Ipl	—	—		
ApC ^c	+ / w +	+ / -		
CpA ^c	+ / w +	+ / -		
UpA ^c	+ / w +	+ / -		
ApA ^c	+ / w +	+ / -		
d(pApT) ^c	+ / w +	+ / -		
d(CpA) ^c	+ / w +	+ / -		
d(ApT) ^c	+ / w +	+ / -		
2',5'-GpA	n	n	+	+
2',5'-ApG	n	n	+	+
ApGpU	n	n	+	+
ApGpCpU	n	n	+	+

^a Symbols used in the table are as follows: +, positive polarization; —, negative polarization; w, weak polarization; n, no observed polarization. d(CpG) and samples with nucleotides containing 5'-phosphates were observed at pH 5; all other samples were measured at pH 7. ^b Polarization of the H8 proton in these dinucleotides is weaker than in other G-containing nucleotides (see text). ^c The intensity and sign of the polarization are dependent on temperature, on buffer phosphate concentration, and on the presence of ionized phosphate groups in the nucleotide (Scheek et al., 1981).

at either 0 or 66 mM phosphate concentration. A shows only very weak polarization in a pH 7 (66 mM phosphate buffer) solution of UpG (2.5 mM), A (2.5 mM), and riboflavin (0.2 mM), whereas A shows strong polarization under identical conditions when UpG is absent [the A(H8) enhancement is less than -0.3 in the former and -6.5 in the latter]. I(H8) and I(H2) both show negative polarization.

G(H8) shows positive polarization in all dimers, although it is much weaker in GpG and d(GpG), likely due to aggregation of the dimers. Except for the latter, the enhancement factor of G(H8) was in the range 11–16 for all dimers. The G(H8) polarization of GpG increases with increasing temperature (20–50 °C), but the enhancement factor is always less than 0.5.

In sharp contrast to the monomers, we observe positive polarization of the G(H1') sugar proton in all dimers (except GpG and dGpG), in the trimer ApGpU (Figure 5), and in the tetramer ApGpCpU (Figure 6). Assignment of the positively polarized resonances as G(H1') follows Lee et al. (1976) and Ezra et al. (1977) for the RNA dimers and Cheng & Sarma (1977) for the DNA dimers. The magnitude of the sugar polarization in GpC or GpU does not change noticeably when G(H8) is saturated with rf during the light pulse or is deuterated. The A or I(H1') protons do not show polarization in the molecules we have studied. The sugar protons of UpG overlap at room temperature but are well resolved at higher temperature; only the H1'-type resonance at higher field is polarized. This polarized resonance also has the larger $J_{1/2}$ coupling constant, consistent with its assignment to the G residue (Ezra et al., 1977). In d(pCpG), the low-field H1' triplet is polarized whereas in d(pGpA) the high-field H1' triplet is polarized. Our assignment of these polarized reso-

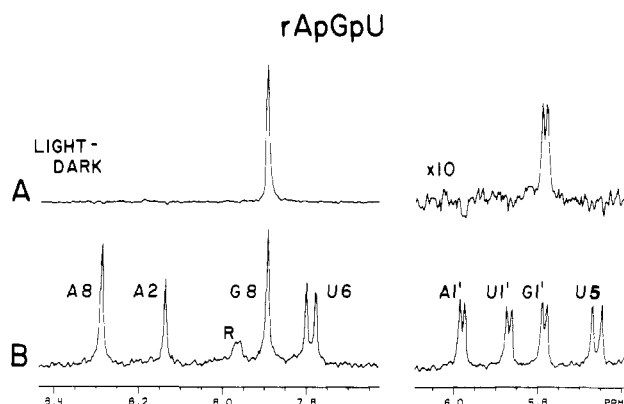


FIGURE 5: ¹H NMR spectra (360 MHz) of ApGpU (1.2 mM) in pH 7 buffer containing 66 mM phosphate and 0.4 mM riboflavin at 22 °C. R indicates riboflavin: (A) light minus dark difference spectrum (four accumulations); (B) dark spectrum (100 accumulations).

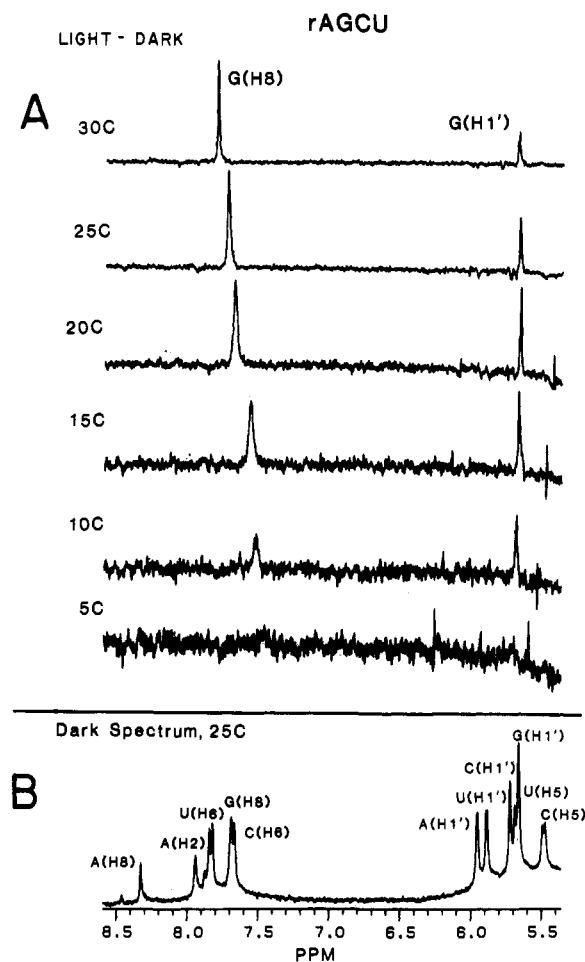


FIGURE 6: ¹H NMR spectra (360 MHz) of ApGpCpU (1.3 mM) in pH 7 buffer containing 5 mM phosphate, 0.2 M NaCl, and 0.4 mM riboflavin. The G(H8) protons were partially deuterated in this sample (obtained from P. Borer). (A) Light minus dark difference spectra at 5, 10, 15, 20, 25, and 30 °C (four accumulations each); (B) dark spectrum at 25 °C (100 accumulations).

nances to the G(H1')'s is consistent with previous assignments for d(CpG) and d(GpA) (Cheng & Sarma, 1977). It is clear that CIDNP offers a very direct approach to assigning G(H1') NMR resonances.

The magnitude of the G(H1') polarization relative to the G(H8) polarization increases with decreasing temperature for GpA, GpU, CpG, dpGpA, ApGpU, and ApGpCpU. We also find that the ratio of the G(H1') polarization to the G(H8) polarization increases in the following series: 0 = monomers

< dimers < ApGpU < ApGpCpU. For the deoxyribo dimers, only the G(H1') to G(H8) polarization ratio for d(GpA) was measured, and the ratio for this compound is similar to that for ApGpU. The ratio of the polarizations for 2',5'-GpA and 2',5'-ApG are similar to or greater than those of the corresponding 3',5' dimers. The T_1 values of G(H8) and G(H1') in GpA at 10, 20, 30, and 40 °C are 1.2 and 2.1, 1.1 and 1.8, 1.3 and 2.6, and 1.4 and 2.7 s, respectively. It is assumed that the T_1 variation as a function of temperature is similar for the other compounds studied. The T_1 values of G(H8) and G(H1') in ApGpU are 1.1 and 1.6 s at 20 °C.

Tetramer. Figure 6 shows a series of CIDNP spectra for the self-complementary oligomer ApGpCpU at different temperatures. The CIDNP intensity of G(H8) decreases dramatically below 20 °C, and essentially no CIDNP is observed below 10 °C. The melting temperature (T_m) of ApGpCpU under these conditions (1.3 mM ApGpCpU, 5 mM phosphate, 0.2 M NaCl, 0.4 mM riboflavin, pH 7) as determined optically is about 17–18 °C and as determined by the average inflection of the proton chemical shift vs. temperature curves is approximately 25 °C. Note that the T_m value for this molecule is strongly dependent on buffer and nucleic acid concentration [compare, for example, Neilson et al. (1980) and Bubenko et al. (1981)]. The chemical shifts of this tetramer are unaffected by the presence of riboflavin at any temperature. GpA and ApGpU were measured in the same buffer system for comparison. The riboflavin chemical shifts and their temperature dependence were different in the presence of ApGpCpU, indicating some effect of the tetramer on the dye. The T_1 values of G(H8) and G(H1') are approximately 1.1 and 1.5 s at 30 °C and 1.0 and 1.6 s at 15 °C in our samples.

Discussion

Mechanism. As in previous studies of the mechanism of the reaction between the flavin triplet state and amino acids (Kaptein, 1980; McCord et al., 1981), we must distinguish between electron transfer and hydrogen-atom abstraction as the primary step. The reaction is clearly the latter for the pyrimidines as no polarization is detected when N1 is substituted. It should be noted that our results for Cyt and Ura differ from those of R. Kaptein and co-workers. They report qualitatively that polarization is not observed for Cyt or for Ura (Kaptein et al., 1979), whereas we observe strong polarization for Cyt and Ura (Figure 3). Hydrogen-atom abstraction is also consistent with the observed polarization of pseudouridine, which retains H1. For Thy, Cyt, and Ura, the radical produced after hydrogen-atom abstraction can be considered an allylic system with large (positive) spin density predicted and observed by EPR at C5 of Cyt and at the 5-methyl protons of Thy and negative spin density at H5 of Ura (Herak & Dulcic, 1972; Zehner et al., 1976; Westhof & van Rooten, 1976). This agrees with the positive CIDNP observed for Thy 5-methyl protons and the negative CIDNP observed for Ura and Cyt 5-protons.

The purines, adenine, guanine, and hypoxanthine, show polarization regardless of the presence or absence of the ribose moiety. It is possible that either the imino or the amino protons are involved in a hydrogen-atom abstraction reaction of the purines. The polarization of m^1G rules out the involvement of the amino protons, assuming the presence of the methyl groups does not alter the mechanism of the reaction. m^1G shows negative polarization of G(H8). This may be due to a change in the mechanism of the reaction but is most likely due to an increase in the g factor of the radical, as discussed below. Thus, it is reasonable to suggest that G protons become

polarized through an electron-transfer mechanism. The electron-transfer mechanism for A is supported by R. Kaptein's studies on the effect of phosphate concentration on the A polarization (Scheek et al., 1981). We have observed no effect of phosphate concentration on the sign of the polarization of G(H8) in ApG or in G. The reaction for I is most likely hydrogen-atom abstraction from N1, as m^1I does not show polarization with any of the dyes we have tried.

The g factors of many of the radicals involved in these reactions have been reported: G^+ , 2.0055 (Dertinger & Hartig, 1972) or 2.0042 (Sevilla & Mohan, 1974); A^+ , 2.0040 (Dertinger & Hartig, 1972) or 2.0043 (Sevilla & Mohan, 1974); Thy $^+$, 2.0053 (Herak & Dulcic, 1972) or 2.0044 (Zehner et al., 1976); flavin anion radical, 2.0034 (Eriksson & Ehrenberg, 1964). The pyrimidine radicals in these studies are believed to be the result of hydrogen-atom abstraction from N1. The reader is cautioned that definitive identification of the nature of the radical is rarely available. The radicals are produced by radiolysis or photolysis in the solid state, the spectra are often poorly resolved, and analyses often depend on calculated spin densities, which may be grossly in error (McCord et al., 1981).

Our CIDNP results are not in agreement with the previously measured g factors for purine radicals given above. This is based on the following analysis. We expect that the g factor of 8BrF is higher than that of riboflavin, due to increased spin-orbit coupling, and that the two flavins react with the bases by the same mechanism. The g factors of the neutral A radical, the I radical, and the m^1G radical must then be in between the g factors of the two flavin radical anions, as the polarizations of these three molecules reverse sign on switching dyes. Using the well-known rules to predict the signs of the polarization (Kaptein, 1971), we can conclude that the spin density at C(8) is positive in the A and G radical cations. This is consistent with most molecular orbital calculations for these radicals (Adams & Box, 1975). We expect that there is positive spin density at C8 in the I radical also, which must have a g factor less than that of the 8BrF radical anion. It is likely that I reacts by hydrogen-atom abstraction of H1, as m^1I does not show polarization with either dye. Thus, our experimental results are consistent with the following ordering of g factors: $A^+ \approx G^+ < \text{riboflavin anion} < m^1G \text{ neutral radical} \approx A \text{ neutral radical} \approx I \text{ neutral radical} < 8\text{BrF anion radical}$.

It is worth noting that the use of several different dye molecules, especially those with quite different g factors, offers several advantages. As the g factors of the target residues (amino acid or nucleotide) are roughly constant, the sign of the spin polarization can be inverted by changing dyes, if this changes the sign of the g factor difference. Furthermore, it is very desirable to perform CIDNP experiments at the highest possible magnetic field strength for maximum resolution; however, the CIDNP intensity decreases with increasing field as the g factor difference (not dependent on nuclear spins) dominates the hyperfine interaction in causing singlet-triplet mixing in the radical pair. This can be offset by using a dye molecule whose g factor is as close as possible to that of the target residue.

Adenine Polarization. The polarization of A is not observed in molecules that also contain G and is greatly attenuated when G-containing molecules are also present in solution; A is strongly polarized in dimers that do not contain G. This effect is likely due to kinetic competition between G and A for the flavin triplet. When the flavin triplet concentration is in excess of the combined concentrations of A and G, both nucleosides

do show polarization. This notion receives some support from EPR studies of nucleoside mixtures in aqueous glasses, from which it has been concluded that the electron-donating ability of G is greater than that of A (Gregoli et al., 1979). Because the A polarization arises from two different radicals with opposite signs for the polarization (Scheek et al., 1981), under certain conditions it is possible for the polarization of A to be zero because the polarization from each pathway exactly cancels. This effect for A itself is due to deprotonation of the A radical cation and is catalyzed by phosphate ions (Scheek et al., 1981). We observe no CIDNP of A in GpA (2.5 mM, 0.4 mM riboflavin) when the phosphate concentration is varied from 5 to 750 mM. It is extremely unlikely that a fortuitous cancellation of A polarization explains its absence when G is present in all of these samples, and we favor the simple kinetic argument. This result has important consequences for the detection of CIDNP in more complex oligonucleotides. Polarization of A will only be observed in the absence of accessible G residues or when the accessibilities of the G residues become sufficiently low to allow an accessible A residue to compete successfully for the photoexcited dye.

Sugar Polarization. Polarization of the G(H1') proton in G-containing dimers and oligomers can result from two possible mechanisms: (1) a finite hyperfine interaction between the H1' nuclear spin and the unpaired electron in the radical intermediate or (2) cross relaxation between another spin-polarized transition and H1' in the diamagnetic product. Cross relaxation is only effective between nuclei with a similar magnetic moment, leaving H8 as the only candidate. Because the polarization is positive for both H1' and H8, dipolar cross relaxation is not likely, as it has been shown that dipolar cross relaxation leads to polarization of the opposite sign for the interacting protons in small molecules (Kaptein, 1979). Consistent with this, we have observed that rf saturation of the H8 resonance during and after the light pulse or deuteration of G(H8) in CpG and UpG do not substantially alter the magnitude of the H1' polarization. Note also that rf saturation of H8 during the light pulse or deuteration of H8 does not result in the appearance of sugar polarization in the monomers 9MeGua, 5'-GMP, or G. Thus, negative polarization due to cross relaxation is not fortuitously cancelling some other source of positive polarization for H1' in these monomers. In the following, we consider five possible mechanisms that could lead to spin density at H1' and might provide a basis for explaining the variations in the magnitude of the H1' polarization.

(1) *Interaction with N9.* Spin density in the aromatic ring can lead to spin density at a β -position by hyperconjugation, σ -bond polarization, and/or an anisotropic dipolar interaction (Gordy, 1980). For aliphatic radicals in solution in which there is unit spin density at the α -carbon, the latter two mechanisms lead to little or no spin density at the β -hydrogen in comparison to the spin density due to hyperconjugation. Spin density due to hyperconjugation between the α -carbon orbital and the C β -H σ -bond has the angular dependence

$$A = (B_0 + B_2 \cos^2 \theta) \rho_\alpha$$

where A is the hyperfine coupling constant, B_0 and B_2 are experimentally determined constants on the order of 0–5 and 40–45 G, respectively (Fischer, 1973), ρ_α is the spin density at the α -position, and θ is the dihedral angle between the α -orbital and the C β -H bond. In the present case, the dihedral angle, θ (the angle between the H1'-C1' bond and the N9 π -orbital), is related to the glycosidic torsion angle, χ (the angle between the O4'-C1' bond and the N9-C4 bond; see Figure 1), by $\theta = \chi - 210^\circ$. The glycosidic torsion angle

decreases when a nucleoside is incorporated in a dinucleotide (Lee et al., 1976; Bangerter & Chan, 1969), increasing the value of $\cos^2 \theta$ and the hyperconjugative spin density on H1'.

Contrary to this analysis, however, the 9-methyl protons of 9MeGua show barely detectable positive polarization. This is not due to an overall lack of reactivity for this derivative, as the H8 polarization is about twice that of the other monomers [this is due, in part, to the substantially longer T_1 of 9MeGua(H8)]. The average hyperfine coupling constant for a proton in a freely rotating methyl group is comparable to or greater than that of a single proton at any fixed angle.

(2) *Opposing Effects.* It is possible that σ -bond polarization and hyperconjugation lead to opposite polarizations at H1'. We do not view this as likely, as it requires two mechanisms of opposite sign, which fortuitously cancel in a number of molecules.

(3) *Interaction with Spin Density at N3.* Baudet et al. (1962) have calculated the spin density in the guanine cation radical and concluded that the spin density at the N9 position is zero, while that at the N3 position is 0.324, far larger than that at any other position in the radical. In order for this mechanism to be selective for the dimers, the H1' in dimers must be closer on average to N3 than in 9MeGua or G. Obviously, the closest possible approach of a proton to N3 in all of these compounds is the same; however, the average distance may be quite different. For 9MeGua, this is the result of free rotation; similarly for G, with the additional note that the preferred syn conformation places the H1' almost as far from N3 as possible. The efficacy of this mechanism, then, depends explicitly on the likelihood that H1' in the dimers and higher oligomers spends more time close to N3 than in a model monomer (see below).

(4) *Dual or Sequential Radical Pairs.* It is conceivable that the radical intermediate for the monomers is different from that of the dimers and that these radicals have very different spin densities at N9. For example, the radical cation might be stabilized in a stacked dimer, whereas the monomer could tend to deprotonate or react via hydrogen-atom abstraction.

(5) *Sugar-Centered Radical.* We view this possibility as highly unlikely for several reasons. (i) Only G sugar protons show polarization; (ii) G in deoxy dimers also shows H1' polarization (no 2'-hydroxyl). (iii) Polarization is never observed or expected for primary and secondary alcohols reacting with the triplet state of riboflavin, and ribose itself shows no polarization.

On balance we favor mechanism three, which suggests that spin density at H1' in dimers and higher oligomers is a consequence of geometrical constraints. Nucleic acid stacking interactions are thought to increase as the length of the oligomer increases and also as the temperature is lowered. In a stacked structure, the anti conformation of the glycosidic angle, χ , is preferred, which brings H1' near to N3, whereas monomers appear to prefer a syn conformation (see Figure 4). The ratio of the enhancement of the G(H1') to that of the G(H8) increases as the length of the oligomer increases and as the temperature is lowered (for example, see Figure 6). The difference in the temperature dependences of the T_1 's for G(H8) and G(H1') in ApG is probably not sufficient to lead to the change in the G(H8) to G(H1') polarization ratio as a function of temperature, nor is the difference in the T_1 's of 9MeGua, G, ApG, and ApGpU at 20 °C sufficient to explain the variation in the amount of sugar polarization among these molecules. Obviously, in taking a ratio, a change in either the G(H8) or the G(H1') polarization intensity affects the ratio; as there is no obvious change in electronic structure,

we assume that it is the H1' spin density that is changing [i.e., G(H8) is an internal standard that corrects for any changes in kinetics and the like with temperature]. It has been suggested from T_1 measurements that deoxyribo dimers are more stacked than the corresponding ribo dimers (Ts'o et al., 1975); consistent with these expectations, d(GpA) has a higher G-(H1') to G(H8) polarization ratio than does GpA.

The 2',5' dimers, however, introduce a complication. We find that 2',5'-GpA, 2',5'-ApG, and 3',5'-GpA have very similar G(H8) enhancement factors and a similar G(H1') to G(H8) polarization ratio. However, 2',5' dimers are thought to have somewhat greater base-base interactions than their 3',5' analogues, on the basis of NMR chemical shift data and hypochromicity measurements (Kondo et al., 1970). CD data demonstrate that 2',5'-GpA is stacked to an appreciable extent at room temperature, but that the stacking geometry differs from that of the 3',5' isomer (Warshaw & Cantor, 1970). In 2',5' dimers, the glycosidic bond angle, χ , of the 2'-residue is thought to be substantially different ($\approx 130^\circ$, low syn) than the 3',5' analogue, on the basis of calculations of the chemical shifts of 2',5'-ApA, 2',5'-ApC, and 2',5'-CpA (Dhingra & Sarma, 1978). If correct, the 130° value would argue against the third, and most promising, mechanism discussed above, as H1' would not be very close to N3. It is evident, however, that conclusions based on chemical shift calculations are much less reliable than those based on direct coupling constant measurements, so it is quite possible that this analysis is in error.

In order to provide further support for mechanism three above, we studied a number of molecules in which the angle χ is constrained. When H1' is at its closest approach to N3, $\chi = 240^\circ$. The glycosidic torsion angle in the model compound V1 is fixed in the syn conformation ($\chi \approx 25^\circ$; as estimated from molecular models, H1' is near to H8). We observe negative H1' polarization and no polarization for H1' if H8 is saturated with rf during the light pulse or deuterated. Thus, the negative polarization of H1' in this molecule is clearly the result of dipolar cross relaxation from H8, and this source of negative polarization is not competing with some other source of positive polarization (spin density at H1' through interaction with N9, N3, or σ -bond polarization) in this molecule. In compound S3, $\chi \sim 275\text{--}305^\circ$; this molecule retains considerable flexibility. In this compound, H1' shows weak negative polarization. In light of the strong H2' polarization, the H1' polarization is probably due to spin density at C2, transmitted to H1' through a σ -bond polarization mechanism. The strong polarization of H2' indicates that the radical of this molecule may well have a different spin density distribution from that of the G radical, and thus is not a good model for our purposes. Compounds S1 and S2 have H1' near to N3 ($\chi \sim 180\text{--}210^\circ$). These compounds showed negligible polarization at pH 7; however at higher pH, they exhibit strong positive polarization of several protons, including positive polarization of H1'. These are the only monomers observed to show appreciable positive polarization of H1'. Observation of positive polarization for the H5' and H5'' in S1 and S2 is consistent with positive spin density at C8 and suggests that the radicals of these molecules are similar to the G radical. To investigate the effect of the amino group at the C8 position on spin density, we have looked at the polarization in adenine and 8-(aminomethyl)adenine [8-(aminomethyl)guanosine was not readily available]. The presence of the amino group at the H8 position does not change the sign of the spin density. Observation of H1' polarization in S1 and S2 supports mechanism three discussed above: the sugar polarization is due to the proximity of H1' to N3.

Tetramer. The reaction leading to the production of CIDNP requires accessibility of the base to the photoexcited flavin. Therefore, it is likely that CIDNP will be observed only in single-stranded oligonucleotides. The temperature dependence of the G(H8) enhancement factor of ApGpCpU (Figure 6) as compared to that of G, GpA, or ApGpU provides a good example of this effect. The T_m of ApGpCpU under these conditions is about 20°C , and a substantial drop in the CIDNP intensity is observed in this molecule as the temperature is decreased below the T_m ; the polarization at 5°C is less than 15% of that at 20°C . This is in contrast to G, GpA, and ApGpU; in these compounds the CIDNP intensity of G(H8) at 5°C ranges from 55 to 85% of that at 20°C . This reduction for G, GpA, and ApGpU is probably the result of changes in T_1 's, diffusion rates, radical lifetimes, exchange rates, aggregation, etc. However, it is clear that the intensity decrease in the CIDNP of G(H8) in ApGpCpU as a function of temperature is much larger than that of G, GpA, or ApGpU, and this is most likely due to inaccessibility of the bases in the double-stranded molecules. The temperature dependence of the ratio of the polarization of H1' to that of H8 is especially obvious for this molecule (Figure 6).

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Registry No. V1, 88979-73-1; S1, 88980-19-2; S2, 88916-33-0; S3, 79483-44-6; 8BrF, 40371-66-2; Ura, 66-22-8; Thy, 65-71-4; Cyt, 71-30-7; 5MeCyt, 554-01-8; A, 58-61-7; 3'-AMP, 84-21-9; 5'-AMP, 61-19-8; 5'-dAMP, 653-63-4; G, 118-00-3; 3'-GMP, 117-68-0; 5'-GMP, 85-32-5; 9MeGua, 5502-78-3; I, 58-63-9; 3'-IMP, 572-47-4; m¹G, 2140-65-0; m²G, 2140-67-2; Gm, 2140-71-8; Ψ , 1445-07-4; m⁷G, 20244-86-4; CpG, 2382-65-2; GpA, 6554-00-3; ApG, 3352-23-6; GpC, 4785-04-0; GpU, 4785-07-3; UpG, 3474-04-2; GpG, 3353-33-1; d-(CpG), 15178-66-2; d-(TpG), 4251-20-1; d-(pCpG), 15623-43-5; d-(pGpG), 26467-04-9; d-(pGpA), 38665-19-9; IpI, 32452-39-4; ApC, 4833-63-0; CpA, 2382-66-3; UpA, 3256-24-4; ApA, 2391-46-0; d-(pApT), 2147-15-1; d-(CpA), 4624-07-1; d-(ApT), 23339-47-1; 2',5'-GpA, 6918-37-2; 2',5'-ApG, 22976-82-5; ApGpU, 3494-34-6; ApGpCpU, 55048-61-8; 2-aminopurine, 452-06-2; 8-(methylamino)adenosine, 13389-13-4; riboflavin, 83-88-5.

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