Conformational Properties of Branched RNA Fragments in Aqueous Solution†

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ABSTRACT: The conformational properties of branched trinucleoside diphosphates A^{C}_{C}, A^{C}_{G}, A^{G}_{C}, A^{G}_{G}, A^{U}_{U}, A^{G}_{U}, A^{U}_{G}, A^{T}_{T}, G^{U}_{U}, and aA^{U}_{U} [X^{Y}_{Z} = X(2′p5′Y)3′p5′Z] have been studied in aqueous solution by nuclear magnetic resonance (^{1}H, ^{13}C), ultraviolet absorption, and circular dichroism. It is concluded from these studies that the purine ring of the central residue (X; e.g., adenosine) forms a base–base stack exclusively with the purine or pyrimidine ring of the 2′-nucleotidyl unit (Y; 2′-residue). The residue attached to the central nucleoside via the 3′–5′-linkage (Z; 3′-residue) is "free" from the influence of the other two heterocyclic rings. The ribose rings of the central nucleoside and the 2′- and 3′-residues exist as equilibrium mixtures of C2′-endo (^{2}E)–C3′-endo (^{3}E) conformers. The furanose ring of the central nucleoside (e.g., A) when linked to a pyrimidine nucleoside via the 2′–5′-linkage shows a higher preference for the ^{2}E pucker conformation (e.g., A^{U}_{G}, A^{U}_{U}, A^{C}_{G}, ca. 80%) than those linked to a guanosine nucleoside through the same type of bond (A^{G}_{U}, A^{G}_{G}, A^{G}_{C}, ca. 70%). This indicates some correlation between nucleotide sequence and ribose conformational equilibrium. The ^{2}E–^{3}E equilibrium of 2′-pyrimidines (Y) shows significant, sometimes exclusive, preference (70–100%) for the ^{3}E conformation; 3′-pyrimidines and 2′-guanosines have nearly equal ^{2}E and ^{3}E rotamer populations; and the ribose conformational equilibrium of 3′-guanosines shows a preference (60–65%) for the ^{2}E pucker. Conformational properties were quantitatively evaluated for most of the bonds (C4′–C5′, C5′–O5′, C2′–O2′, and C3′–O3′) in the branched "trinucleotides" A^{U}_{U} and A^{G}_{G} by analysis of ^{1}H–^{1}H, ^{1}H–^{31}P, and ^{13}C–^{31}P coupling constants. The C4′–C5′ bond of the adenosine units shows a significant preference for the γ^{+} conformation. The dominant conformation about C4′–C5′ and C5′–O5′ for the 2′- and 3′-nucleotidyl units is γ^{+} and β^{t}, respectively, with larger γ^{+} and β^{t} rotamer populations for the 2′-unit. The increased conformational purity in the 2′-residue, compared to the 3′-residue, is ascribed to the presence of an ordered (adenine ⇔ 2′-residue) stacked state. The favored rotamers about C3′–O3′ and C2′–O2′ are ϵ^{-} and ϵ'^{-}, respectively. The conformational features of A^{U}_{U} and A^{G}_{G} were compared to those of their constitutive dimers A3′p5′G, A2′p5′G, A3′p5′U, and A2′p5′U and monomers 5′pG and 5′pU.

Mammalian pre messenger RNA (pre-mRNA) splicing occurs by two kinetically distinct reaction steps (Padgett et al., 1984; Ruskin et al., 1984; Domdey et al., 1984). First, the pre-mRNA is cleaved at the 5′ splice site, generating the 5′-exon and an RNA species composed of an intervening sequence (IVS, or intron) attached to the 3′-exon (IVS–3′-exon species). Second, cleavage at the 3′ splice site and ligation of the 5′- and 3′-exons occur, resulting in the excission of the intact intron. The excised intron and the IVS–3′-exon intermediate are in the form of a lariat in which the 5′-end of the intron is joined by a 2′–5′-phosphodiester linkage to an adenosine residue near the 3′-end of the intron. The structure of the branch is A(2′p5′G)3′p5′C (A^{G}_{C}) in the case of β-globin (Ruskin et al., 1984) and yeast RNA (Domdey et al., 1984) and A(2′p5′G)3′p5′U (A^{G}_{U}) in adenovirus 2 transcripts (Padgett et al., 1984).

Recently, it has been demonstrated that the efficiency of the messenger RNA splicing reaction is influenced by the sequences at the 5′-(A-G:*G-U-A-A-G-U*) and 3′-(*Py11-N-C-A-G*:G) splice sites and the RNA branch point (*Py-N-Py-Pu-A-Py*) (Reed & Maniatis, 1985; Vijayraghan, 1986; Hornig et al., 1986). Mutation of the normal branch-accepting adenosine nucleotide to G in β-globin pre-mRNA does not inhibit branch formation at the mutated position (G^{G}_{C}), but the splicing reaction stops at this stage with accumulation of the mutated lariat IVS–3′-exon intermediate (Hornig et al., 1986). Mutation of the 5′ splice site residue, normally G-U, to A-U, G-A, and G-G also prevents the second step of splicing while allowing the first step (Hornig et al., 1986). These results suggest that sequence changes at either the branch point or the 5′ splice site do not block recognition and cleavage of the 5′ splice site. However, there appears to be a proofreading step in which sequences at the branch site of the lariat IVS-3′–exon intermediate are examined before execution of the second step of the reaction.

It is conceivable that it is not only the primary sequence of the branch point that is essential for the second step of the splicing reaction but that the three-dimensional structure and conformational behavior of the branch may also be significant. The study of the conformational preferences of branched RNA may therefore provide a deeper insight into the mechanisms that govern the splicing process.

In this paper we present a detailed (NMR, UV, CD) study of the conformational properties of a series of branched "trinucleotides" (branched trinucleoside diphosphates A^{C}_{C}, A^{C}_{G}, A^{G}_{C}, A^{G}_{G}, A^{U}_{U}, A^{G}_{U}, A^{U}_{G}, A^{T}_{T}, G^{U}_{U}, and aA^{U}_{U}).[1]

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; ppm, parts per million; X^{Y}_{Z}, the branched nucleotide sequence X(2′p5′Y)3′p5′Z, where X is the branched nucleoside; aA^{U}_{U}, the branched arabinoadenosine diuridine trinucleoside diphosphate; $X_{p}Y$ and $X^{p}Y$, the linear sequences X3′p5′Y and X2′p5′Y, respectively.

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Experimental Procedures

Synthesis of Nucleotides. The branched nucleotides were synthesized and deprotected as previously described (Damha et al., 1985; Damha & Ogilvie, 1988). Dinucleoside monophosphates (A_pU, A^pU, A_pG, A^pG) and trinucleoside diphosphates (U_pA_pU, U_pA^pG, U^pA^pG) were prepared by the β-cyanoethyl phosphodichloridite procedure (Ogilvie et al., 1980).

Hypochromicity Measurements. The hypochromicity values (H) of nucleotides were calculated from the absorption maximum (A) of the ultraviolet spectrum before and after hydrolysis in accordance with

$$H(\%) = [(A_{\text{monomer}} - A_{\text{nucleotide}})/A_{\text{monomer}}] \times 100$$

Hydrolysis of the nucleotides with snake venom phosphodiesterase was employed to determine $H(\%)$. A 100-μL aliquot of the nucleotide in a buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0) was digested by treatment with 10 μL of enzyme solution (1 mg/mL 1:1 glycerol:water) in a sealed 1.5-mL Eppendorf tube at 37 °C for 12–16 h. Another 100-μL aliquot of the nucleotide solution lacking the enzyme was incubated under identical conditions. After incubation, both samples were diluted with 3.0 mL of water and their UV spectra measured against an appropriate blank on a Hewlett-Packard 8451A spectrophotometer. Hypochromicity was within ±3% in duplicates.

Circular Dichroism (CD) and UV. The CD spectrum of each sample was measured on a Jasco 500-C spectropolarimeter with a Jasco DP-J800/300 data processor. The cell temperature was controlled by the use of a Haake constant-temperature circulator (accurate to ±2 °C). A water-jacketed fused quartz window cell (no. 165-QS) of 1-cm path length was obtained from Hellma (Canada) Ltd. (Concord, Ontario). The concentration of the nucleotides employed was 0.5–2.0 A_{260} units (about 10^{-4} M), dissolved in 5 mM Na_2HPO_4 and 100 mM NaCl (pH 7.4). The exact concentrations of these solutions were determined spectrophotometrically. CD results are reported in terms of $[\theta]$ (molar ellipticity) in units of deg M^{-1} cm^{-1} calculated with the formula $[\theta] = 100E/(cl)$, where E is the observed ellipticity angle, l is the cell length, and M is the concentration of the nucleotide in mol/L. The concentration of the nucleotide solution was calculated with the formula, $c = A/[\epsilon_{\text{monomer}} - H(\%)\epsilon_{\text{monomer}}]$. Extinction coefficient values (ϵ) of 14.9, 13.6, 10.1, 9.1, and 9.7 were used for adenosine, guanosine, uridine, cytidine, and thymidine, respectively.

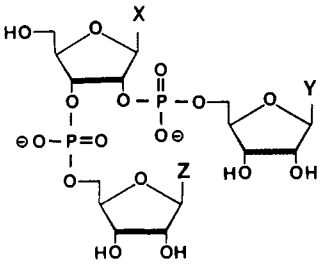
NMR. The NMR samples were prepared as follows: The sodium salts of the nucleotides were dissolved in 0.50 mL of phosphate buffer (5 mM Na_2HPO_4, 100 mM NaCl, 0.1 mM EDTA, pD of buffer adjusted to 7.4 by addition of concentrated HCl) and lyophilized. The samples were then lyophilized twice from 99.8% D_2O (0.25 mL) and once from 99.96% D_2O (0.25 mL) and finally dissolved in 99.96% D_2O (0.50 mL). These solutions were transferred via syringe to 5-mm NMR tubes (Wilmad Glass Co., no. 528PP). The pDs (pH + 0.4) of these solutions were in the range of 7–8. The sample concentrations were in the range of 5–10 mM.

All spectra, except ^1H spectra with specific ^{31}P resonance decoupling (vide infra), were recorded on a Varian XL-300 spectrometer. ^1H and ^{13}C spectra were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, external reference, Aldrich Chemical Co.). ^1H FIDs were multiplied by an appropriate Gaussian function to enhance the spectral resolution, zero filled to 32K data points and Fourier transformed. $^3J_{H-H}$ coupling constants were obtained directly from the observed spectra. When the spectra were not first order, a further refinement was made by the use of the simulation–iteration program LAME in a Varian computer. The samples for the acquisition of ^{13}C NMR spectra were the same as those used to obtain ^1H NMR spectra (ca. 5 mM). Typical acquisition times were 12–16 h (50 000–60 000 scans). Pulse repetition rates were typically 0.9 s, with pulse widths of 65°. ^{13}C NMR spectra were multiplied by an appropriate Gaussian function to enhance the resolution of the C2′ and C3′ resonances of the branched trinucleotides, zero filled to 128K data points and Fourier transformed. ^1H spectra with specific ^{31}P resonance decoupling were obtained with a Brucker WH-400 spectrometer (Université de Montréal, Quebec) equipped with standard Brucker accessories. This method has previously been described (Cheng et al., 1984).

Results and Discussion

Base–Base Stacking

It was of interest to determine whether branched trinucleotides exhibit base stacking similar to that often found in small RNA fragments (Ts'o, 1974; Cantor & Schimmel, 1980) Examination of Corey–Pauling–Koltum (CPK) models of these molecules shows that three base–base interactions are possible in branched trinucleotides (X^Y_Z, **1**): the interaction



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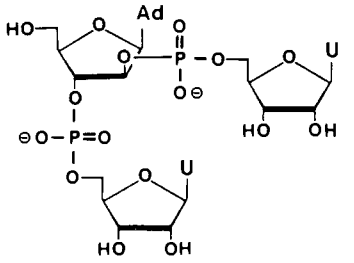

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of the base of the central nucleotide (X) with either the base of the residue linked through the 3′–5′-linkage (i.e., X–Z stacking) or the base of the residue linked through the 2′–5′-linkage (i.e., X–Y stacking). The third possibility for stacking involves the bases of Y and Z (i.e., Y–Z stacking). Study of the models indicates that the interaction of the X and Z bases would be somewhat hindered by the 2′–5′-linked residue (Y). This is especially true in aA^U_U (**2**) in which the 2′–5′-linked nucleotide is positioned on the same side as the central adenine base. The actual interactions between neighboring chromophores in branched trinucleotides were determined as described below.

UV Absorption. It is well established, both experimentally (Michelson, 1963) and theoretically (Tinoco, 1958, 1961; Rhodes, 1961), that stacking of bases leads to hypochromism. The strength of the hypochromism depends on the inverse cube of the distance between the parallel-plane-interacting chro-

Table I: Circular Dichroism, Hypochromicity, and Absorption Spectral Data of Branched and Linear Nucleotides[a] at 22 °C

nucleotide	λ_{max}	$\epsilon \times 10^{-3}$	% *H*	λ_+	λ_0	λ_-	$[\theta]_+ \times 10^{-4}$	$[\theta]_- \times 10^{-4}$
branched								
A^{U}_{U}	261	31.2	11	276	260	245	1.9	−1.8
A^{U}_{G}	257	32.9	15	274	258	243	1.0	−0.9
A^{C}_{C}	264	27.1	18	278	269	252	4.0	−2.2
A^{C}_{G}	258	30.9	18	280	264	257	2.4	−4.1
A^{G}_{G}	254	35.5	16	288	280	266	0.2	−0.6
				243			0.2	
A^{G}_{U}	256	36.8	5	263	241	238	1.2	−0.1
A^{G}_{C}	257	36.6	3	275		234	2.4	0.0
				255[b]			1.2	
G^{U}_{U}	257	28.1	17	265	281	287	3.7	−0.7
					250	242		−1.7
A^{T}_{T}	264	28.5	17	280	268	249	1.7	−1.9
aA^{U}_{U}	261	29.5	16	269	256	241	1.3	−1.9
linear								
A_pU	260	22.5	10	267	257	246	2.4	−1.4
A^PU	262	21.5	14	275	260	248	2.0	−1.7
A_pG	256	26.6	7	279	270	259	1.7	−2.5
A^PG[c]	258	24.6	14	290		~280	0.1	0.0
				252			0.6	
U_pA_pU	260	31.2	11	267	255	245	3.8	−1.7
U_pA^PG	258	33.3	14	272	261	245	1.1	−0.6

[a] Symbols are as follows: λ_{max} is the maximum wavelength in nm; % *H* is the hypochromicity at λ_{max}; ϵ is the extinction coefficient at this wavelength in cm^{-1} mol^{-1} L; λ_+, λ_0, and λ_- are the wavelengths of the peak, crossover, and trough of the circular dichroic spectrum in nm; $[\theta]_+$ and $[\theta]_-$ are the molar ellipticities at λ_+ and λ_0, respectively, in deg cm^{-1} mol^{-1} L. [b] Shoulder. [c] Circular dichroic data obtained from Sussman et al. (1973).

Table II: Base and Anomeric ^1H NMR Chemical Shifts of Free Branched RNA Fragments[a]

	A			3′p5′B			2′p5′C		
	H1′	H2	H8	H1′	H5(2)	H6(8)	H1′	H5(2)	H6(8)
A^{U}_{U}	6.21	8.00	8.31	5.96	5.88	7.88	5.60	5.65	7.36
A^{U}_{G}	6.07	8.03	8.30	5.90		8.02	5.60	5.66	7.37
A^{G}_{U}	6.19	7.86	8.23	5.92	5.84	7.87	5.63		7.72
A^{G}_{G}	6.10	7.79	8.21	5.88		8.01	5.60		7.65
A^{C}_{G}	6.15	7.95	8.30	5.90		8.02	5.55	5.74	7.34
A^{G}_{C}	6.16	7.79	8.22	5.91	5.96	7.84	5.61		7.61
A^{C}_{C}	6.16	7.94	8.28	5.94	6.01	7.88	5.54	5.74	7.34
A^{T}_{T}	6.19	8.00	8.33	6.30	1.85	7.69	6.00	1.78	7.23
G^{U}_{U}	6.06		7.99	5.97	5.92	7.93	5.71	5.79	7.69
aA^{U}_{U}	6.50	8.16	8.30	5.92	5.88	7.89	5.69	5.71	7.44

[a] Sample conditions described under Experimental Procedures.

mophores. The branched trinucleotides were degraded with snake venom phosphodiesterase, and the UV spectrum was measured in H_2O before and after the enzyme digestions. The branched trinucleotides A^{C}_{C}, A^{C}_{G}, A^{G}_{C}, A^{G}_{G}, A^{U}_{U}, A^{G}_{U}, A^{U}_{G}, A^{T}_{T}, G^{U}_{U}, and aA^{U}_{U} all absorbed less UV light (i.e., hypochromism) than the corresponding degradation products (nucleoside and nucleotide monomers), which is indicative of a locally stacked arrangement of the bases in the trimer. The hypochromicity values (% *H*) of these derivatives at room temperature are collected in Table I. Hypochromicity values ranged from 3% to 18% and are similar to % *H* values obtained for dinucleoside monophosphate (Warsaw & Tinoco, 1966) and trinucleoside diphosphate (Cantor & Tinoco, 1965) RNA fragments. The % *H* values of the dinucleoside monophosphates A2′p5′U (A^PU), A3′p5′U (A_pU), A^PG, and A_pG and the linear trinucleotides U_pA_pU and U_pA^PG were also determined and are shown in Table I for comparison. The % *H* values of the four dinucleoside monophosphates that we used as reference compounds agree, within experimental error, with the results of Lapidot et al. (1963). The naturally occurring branched trinucleotides A^{G}_{U} and A^{G}_{C} showed low % *H* values (5% and 3%, respectively), indicating a small, but definite, degree of base–base interaction. NMR and CD measurements indicate that A^{G}_{U} and A^{G}_{C} have a locally stacked arrangement of the bases.

NMR. (A) Chemical Shifts. The spectral data and assignment of the ^1H, ^{13}C, and ^{31}P resonances of the branched trinucleotides have been reported elsewhere (Damha & Ogilvie, 1988). The base and anomeric ^1H NMR data of the branched trinucleotides are presented in Table II. The following general characteristics are observed:

(i) Without exception, the nucleotide residue attached to the central nucleoside via the 2′–5′-linkage (2′-residue) exhibits ^1H (^{31}P and ^{13}C) resonances at higher field than that attached through the 3′–5′-linkage (3′-residue).

(ii) The base and anomeric proton chemical shift (δ) values of the 2′-residues are primarily influenced by the nature of the central nucleoside (i.e., adenosine or guanosine) and vice versa. For example, the chemical shifts of the protons of adenine and 2′-guanine show very little variation over the series A^{G}_{C}, A^{G}_{U}, and A^{G}_{G}. On the other hand, the chemical shifts of the 2′-uracil protons in A^{U}_{U} and G^{U}_{U} differ substantially.

(iii) The base and anomeric proton δ values of a given 3′-residue are nearly independent of the nature of both the central nucleoside and the 2′-residue.

These observations strongly suggest that an intramolecular base–base interaction occurs between the base of the central nucleoside (i.e., adenine or guanine) and the base of the 2′-residue.

The small dependence of the base proton chemical shifts of the 3′-residue on the nature of the central nucleoside and 2′-residue suggests that this residue does not participate in any ring–ring interaction and that it is positioned far away from the stack. For example, the chemical shifts of the 3′-guanosine

Table III: ^{1}H Dimerization [δ(Monomer − Dimer)] and Trimerization [δ(Monomer − Trimer)] Shifts (ppm) at 20 °C[a]

nucleotide		1′	2′	3′	4′	5′	5″	2(5)	8(6)	Δ[b]
A_pU	-pU	−0.26	~−0.10	~−0.14	~−0.03	0.14	0.01	−0.35	−0.28	0.13
A^pU	-pU	−0.40	−0.34	−0.39	−0.40	−0.24	−0.42	−0.32	−0.62	0.18
A^U_U	-2′pU	−0.41	−0.38	−0.43	−0.45	−0.29	−0.57	−0.29	−0.63	0.28
	-3′pU	−0.04	0.01	−0.03	0.01	0.12	0.09	−0.06	−0.11	0.03
A_pG	-pG	−0.17	−0.06	−0.01	−0.05	0.11	0.02		−0.22	0.08
A^pG	-pG	−0.38	−0.30	−0.21	−0.31	−0.21	−0.33		−0.42	0.12
A^G_G	-2′pG	−0.34	−0.26	−0.27	−0.39	−0.28	−0.56		−0.45	0.28
	-3′pG	−0.06	0.13	0.06	0.00	0.06	0.08		−0.10	−0.02

[a] The chemical shift data of uridine and guanosine 5′-monophosphate monomers (pD 5.5, 10–30 mM) were obtained from Lee et al. (1976). [b] $\Delta = (\delta_{5'} - \delta_{5''})_{dimer} - (\delta_{5'} - \delta_{5''})_{monomer}$ in the case of dinucleoside monophosphates and $(\delta_{5'} - \delta_{5''})_{trimer} - (\delta_{5'} - \delta_{5''})_{monomer}$ in the case of branched trinucleotides.

Table IV: ^{1}H NMR Chemical shifts[a] at 22 °C of Branched Trinucleotides A^U_U and A^G_G and Dinucleoside Monophosphates A^pU, A_pU, A^pG, and A_pG in D_2O[b]

nucleotide		1′	2′	3′	4′	5′	5″	2(5)	8(6)
A^U_U	A	6.19	5.20	4.89	4.58	3.89	3.83	8.00	8.31
	-2′pU	5.58	3.98	3.96	3.83	3.87	3.52	5.65	7.36
	-3′pU	5.95	4.37	4.36	4.29	4.28	4.18	5.88	7.88
A^pU	A	6.19	5.09	4.62	4.36	3.92	3.84	8.04	8.33
	-pU	5.59	4.02	4.00	3.88	3.92	3.67	5.62	7.37
A_pU	A	6.02	4.84	4.63	4.44	3.97	3.86	8.14	8.30
	-pU	5.73	~4.25	~4.25	~4.25	4.30	4.10	5.59	7.71
A^G_G	A	6.10	5.20	4.89	4.41	3.73	3.69	7.79	8.21
	-2′pG	5.60	4.39	4.21	3.96	3.90	3.56		7.65
	-3′pG	5.88	4.78	4.54	4.35	4.24	4.20		8.00
A^pG	A	6.14	5.10	4.63	4.23	3.82	3.70	7.84	8.15
	-pG	5.56	4.35	4.27	4.04	3.97	3.79		7.68
A_pG	A	5.89	4.69	4.63	4.32	3.82	3.76	8.04	8.19
	-pG	5.77	4.59	4.47	4.30	4.28	4.14		7.88

[a] Shifts are given in ppm relative to external DSS and are accurate ±0.01 ppm. [b] pD 7–8; concentration 5–10 mM.

nucleoside in A^C_G, A^U_G, and A^G_G are almost identical. Similarly, the base proton chemical shifts of the 3′-uridine residues in G^U_U, A^U_U, aA^U_U, and A^G_U are virtually identical.

(B) Dimerization and Trimerization Effects. To further investigate the ring–ring interactions in the branched trinucleotides, we compared the NMR (and CD, vide infra) properties of A^U_U with those of the dinucleoside monophosphates A2′p5′U (A^pU) and A3′p5′U (A_pU). In a crystal structure of A^pU the bases are stacked in a right-handed helix and overlapped slightly, with an average interplanar distance of 0.34 nm (Shefter et al., 1964, 1969). Sussman et al. (1973) and Doornbos et al. (1981) studied the conformation of A^pU in aqueous solution by means of CD and NMR and came to the conclusion that this dimer has a substantial degree of base stacking in solution. Similarly, the base residues of the 3′–5′-linked isomer A_pU stack appreciably both in solution (Brahams et al., 1967; Erza et al., 1977) and in the crystal (Seeman et al., 1976).

One procedure successfully adopted for the studies of base–base interaction in dinucleoside monophosphates, e.g., A^pU, is to compare the ^{1}H δ values of the 2′-residue (−pU) in the dimer with the δ values of the corresponding nucleotide 5′-monophosphate monomer (pU). This difference, $\Delta_d = \delta_{dimer} - \delta_{monomer}$, usually negative, is termed dimerization shift and reflects the shielding of protons by the neighboring nucleotidyl unit in the dimer (Ts'o et al., 1969; Lee et al., 1976; Ezra et al., 1977). Table III shows the dimerization shift values of the uridine residues in A_pU and A^pU and the trimerization shift values, $\Delta_t = (\delta_{monomer} - \delta_{trimer})$, of both uridine units in A^U_U. It is clear from Table III that Δ_d and Δ_t values of the 2′-residues in each sample are very similar (i.e., the chemical shifts of the 2′-uridine residues in A^pU and A^U_U are similar; Table IV, Figure 1). These results strongly suggest that the base–base stacking between the adenine and the 2′-uracil bases in A^pU is preserved in A^U_U and, on the basis of the Δ_d and

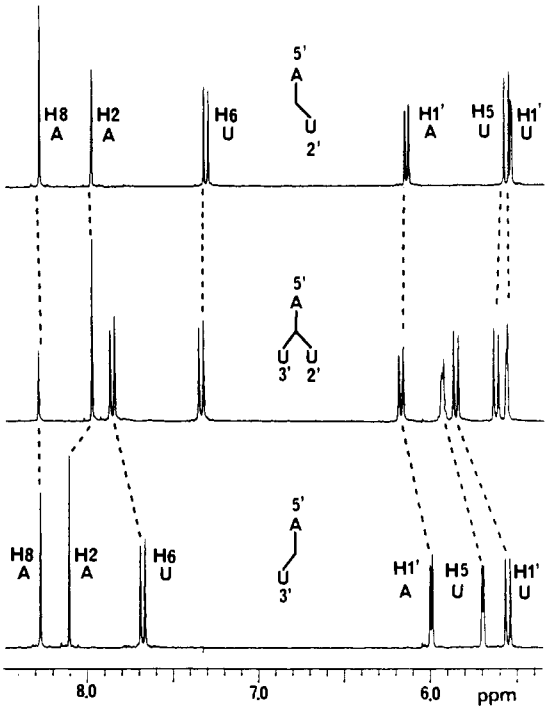

FIGURE 1: ^{1}H NMR (300 MHz) spectra of A^pU, A^U_U, and A_pU at 20 °C (base and anomeric region).

Δ_t data, the extent of base overlap appears to be somewhat greater in the latter. With a few exceptions, the Δ_d values of the 2′-uridine residue are slightly larger (less negative) than the corresponding Δ_t values. Since the chemical shift observed for a specific proton represents a time-averaged value for stacked and unstacked species, the above observation could reflect a small increase in the population of base-stacked

Table V: ^1H $\Delta\delta_T$ Values[a] (ppm) for the Base and Ribose Protons of A^U_U, A^pU, and A_pU and A^G_G, A^pG, and A_pG

nucleotide		1′	2′	3′	4′	5′	5″	2(5)	8(6)
A^U_U	A	−0.06	−0.13	−0.07	−0.02	−0.05	−0.07	−0.17	−0.04
	−2′pU	−0.14	−0.10	−0.08	−0.07	−0.07	−0.12	−0.10	−0.12
	−3′pU	−0.05	−0.02	−0.03	−0.03	−0.02	−0.05	−0.06	−0.02
A^pU	A	−0.07	−0.11	−0.05	−0.04	−0.06	−0.07	−0.18	−0.05
	−pU	−0.15	−0.08	−0.09	∼−0.08	∼−0.04	−0.10	−0.11	−0.12
A_pU	A	−0.08	−0.05	−0.12	−0.05	−0.01	−0.03	−0.12	−0.05
	−pU	−0.14	∼−0.05	∼−0.05	∼−0.05	∼0.00	−0.07	−0.19	−0.08
A^G_G	A	−0.14	−0.17	−0.10	−0.12	−0.12	−0.16	−0.29	−0.10
	−p2′G	−0.14	−0.14	−0.07	−0.09	−0.05	−0.16		−0.16
	−p3′G	−0.07	−0.05	−0.05	−0.06	−0.09	−0.08		−0.08
A^pG	A	−0.08	−0.08	−0.02	−0.08	−0.09	−0.12	−0.23	*b*
	−pG	−0.14	−0.14	−0.05	−0.06	−0.04	−0.05		−0.13
A_pG	A	−0.13	−0.09	−0.11	−0.03	−0.03	−0.02	−0.19	−0.03
	−pG	−0.11	−0.12	−0.04	−0.06	∼0.00	−0.06		−0.10

[a] $\Delta\delta_T = \delta(20°) - \delta(70°)$. [b] H8 signal exchanged with deuterium.

species in the trimer. The hypochromicity values of A^pU and A^U_U, however, indicate that, within experimental error, these nucleotides have the same degree of stacking. In contrast, Δ_d and Δ_t values of the 3′-units show considerable variation in the different molecules. The very small Δ_t values of the 3′-uridine (A^U_U) indicate that this residue is totally free from the influence of the other bases and has an environment very similar to uridine 5′-monophosphate. Similar results were obtained in the analysis of dimerization and trimerization shifts of A_pG, A^pG, and A^G_G (Table III).

Another index of stacking is provided by the chemical shift difference (magnetic nonequivalence) of H5′ and H5″ of the 2′- and 3′-residues relative to that in the monomer; i.e., $\Delta = (\delta_{5''} - \delta_{5'})_{dimer} - (\delta_{5''} - \delta_{5'})_{monomer}$ and $\Delta = (\delta_{5''} - \delta_{5'})_{trimer} - (\delta_{5''} - \delta_{5'})_{monomer}$. The δ values of A_pU, A^pU, A^U_U, A_pG, A^pG, and A^G_G are listed in Table III. The difference in the magnetic nonequivalence between the H5′ and H5″ protons of the uridine residue (2′-uridine in the case of A^U_U) increases in the order pU < A_pU < A^U_U (Table III). Similarly, for the guanosine residues (2′-guanosine in the case of A^G_G) this difference increases in the sequence pG < A^pG < A^G_G. The progressive loss of magnetic equivalence of H5′ and H5″ of the 2′-uridine and guanosine residues may reflect the "locking in" of the 2′-phosphate backbone torsional freedom due to a progressive increase in the population of the base-stacked species. This is in contrast to the behavior of the δ values of H5′ and H5″ of the 3′-uridine and guanosine residues. Upon dimerization Δ increases to about 0.20–0.30 ppm but drops back to 0 upon trimerization. With Δ a monitor of stacking (Lee et al., 1976) we can conclude that the 2′-uridine and guanosine residues in A^U_U and A^G_G are, on average, base stacked with the adenine moiety whereas the 3′-residues are not. This is in agreement with the other spectroscopic results described above.

(C) Temperature Dependence of the ^1H Chemical Shifts. The temperature dependence (20–75 °C) of the chemical shifts of the base (H5, H6) and sugar protons (H1′, H5″) of both uridine residues in the trinucleotide A^U_U (5 mM D_2O) is shown in Figure 2 and Table V. The results indicate that there is a more profound temperature effect on the chemical shifts of H6, H5, H1′, and H5″ of the 2′-uridine nucleotide than of the 3′-uridine fragment. The chemical shifts of the adenine protons, in particular H2 and H2′, were also very sensitive to temperature variation. These results are consistent only with a base–base overlap between the adenine ring and the 2′-uracil base. The dinucleoside monophosphates A_pU, A^pU, A_pG, and A^pG also exhibit decreased stacking as the temperature increases (Table V).

The temperature effect on A^U_U (adenosine and 2′-uridine)

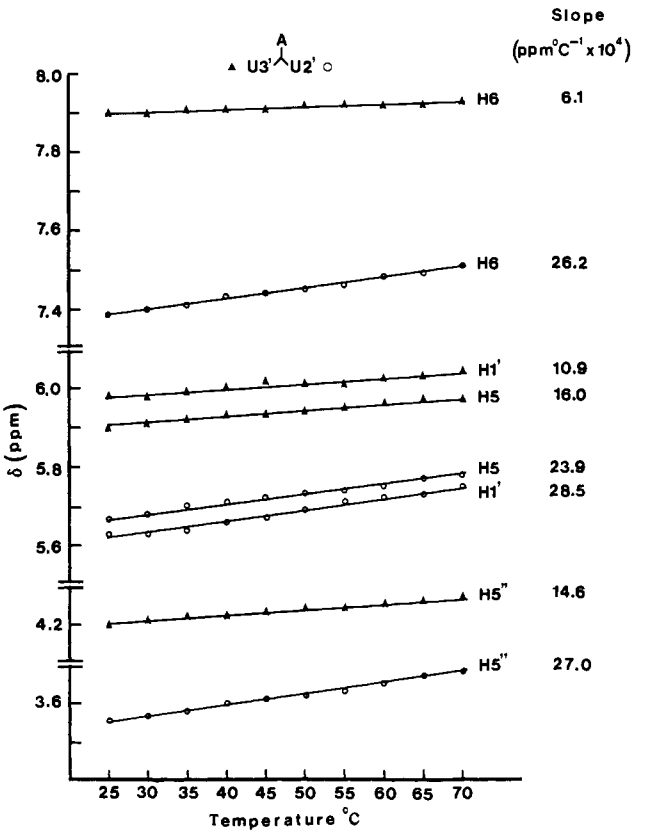

FIGURE 2: Temperature dependence of the chemical shifts of the base and ribose H1′, H5″ protons of the uridine residues of A^U_U (D_2O, pD 7–8).

is virtually the same as for A^pU. This clearly suggests that the chemical environment and conformational behavior of the adenosine and 2′-uridine residues in A^U_U and A^pU are very similar. On the other hand, the 3′-uracil residues in A^U_U and in A_pU behave quite differently from each other. The small deshielding effects observed for the 3′-uracil protons in A^U_U with increasing temperature can be rationalized by an absence of base–base interaction (of adenosine and the 3′-residue) as compared to A_pU.

Circular Dichroism (CD). Figure 3 shows the CD spectra of the branched trinucleotides and the corresponding 2′–5′- and 3′–5′-linked dinucleoside monophosphate compounds. The CD spectra of A^pU and A_pU agree very well with the results of Sussman et al. (1973) and Doornbos et al. (1981). The CD spectrum of A^pU differs in peak position and in magnitude from that of A_pU, and this is attributed to conformational differences between the two compounds (Sussman et al., 1973).

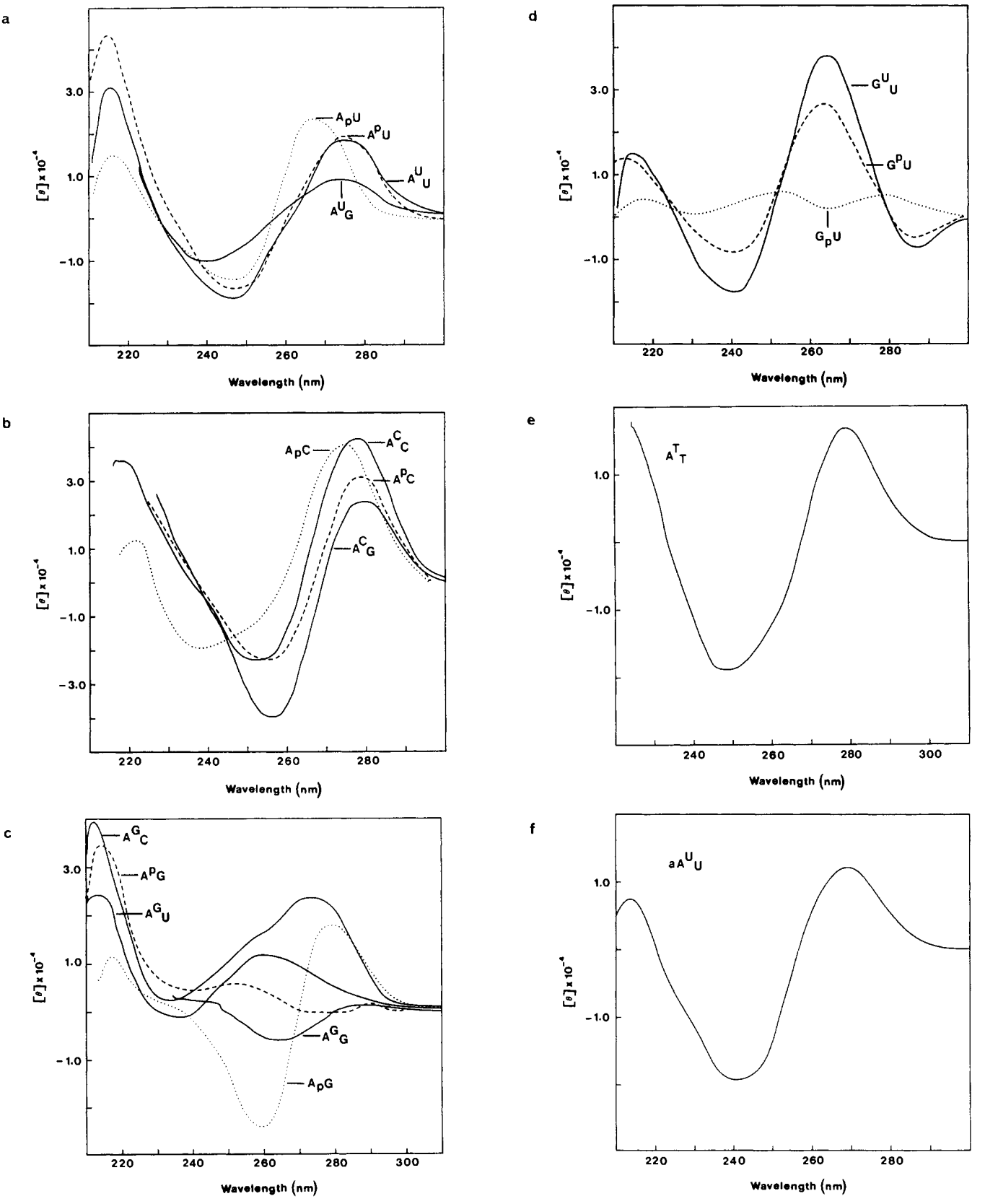

FIGURE 3: CD spectra of dinucleoside monophosphates and branched trinucleotides in H_2O at 22 °C (pH 7.4). The spectra of G^pU, G_pU, A^pC, A_pC, and A^pG were obtained from Sussman et al. (1973).

It is clear from Figure 3a–d that the CD patterns (peak, trough, and crossover positions) of a given branched trinucleotide, X^Y_Z, match very well with the CD patterns of its corresponding 2′–5′ dinucleoside monophosphate compound, X^pY. For instance, the CD spectra of A^U_U, A^U_G, and A^pU show long-wavelength positive Cotton effects at 276, 274, and 275 nm and negative Cotton effects at 245, 243, and 248 nm, with intersection points at 260, 258, and 260 nm, respectively (Figure 3a). The Cottons effects are situated at a wavelength very near the absorption maximum (261, 257, and 260 nm, respectively). Similarly, the CD of G^U_U showed peak, trough, and crossover positions similar to those of G^pU but different

from those in the CD spectrum of G_pU (Figure 3d).

The characteristic double Cotton effects in the CD of A^U_U, A^U_G, A^C_C, and A^C_G are in agreement with the exciton theory of polynucleotides (Tinoco, 1964), which predicts that the interactions among identical chromophores (and, in some cases, among different bases; Bush, 1974) in a dimer will give rise to the splitting of the monomer band into two bands (exciton splitting). It was also observed that the sum of the rotational strengths (peak area of Cotton effects) for these molecules is approximatively equal to zero in the spectral region under consideration ("conservative" optical activity).

In contrast to the above molecules, the optical activity derived from the interaction of the chromophores in A^pG, A^G_G, A^G_C, A^G_U, and G^U_U is *not* of the conservative type. The CD spectra of A^pG, A^G_C, and A^G_U are composed of one main positive band centered at 255, 275 (255 sh), and 263 nm, respectively, while the CD spectrum of A^G_G is composed of a negative band at 265 nm and a positive band at 288 nm of relatively weak intensity (Figure 3c). Nonconservative CD spectra are usually exhibited by base-stacked dimers containing guanine bases (Brahams et al., 1967; Warsaw & Cantor, 1970). This is thought to be due to the presence of two or more overlapping π–π^* bands in guanine monomers in the spectral region under consideration (Williams & Moore, 1983; a rare exception is the CD of A_pG, which is conservative; Figure 3c). While this makes interpretation of the CD spectra of guanine-containing nucleotides very difficult, in general, it gives confidence that the guanine residues in the molecules of this study (A^G_G, A^G_C and A^G_U, and G^U_U) are involved in a stacking interaction, in agreement with the NMR studies discussed above.

The critical aspect of the structure for CD (peak and trough positions) is the relative orientation and proximity of the two base chromophores, which are affected by the conformation of the phosphodiester linkages and asymmetric sugar residues to which the bases are attached. Since the CD patterns of branched trinucleotides are very similar to those of their constitutive 2′–5′ dinucleoside monophosphate compounds, we can conclude that the relative orientation and proximity of the central base and 2′-base in these molecules are similar; i.e., *stacking of bases in branched trinucleotides* (X^Y_Z) *occurs between the central base and the base of the 2′-residue* (X and Y, respectively).[2]

The CD spectra of aA^U_U and A^T_T also consist of a pair of adjacent positive and negative conservative bands with the intersection point situated at a wavelength near the absorption maximum, indicating that these nucleotides also exhibit base–base stacking interactions (Figure 3e,f). As will be shown from the following studies, the exciton splitting observed in the CD spectra of these trimers is due to the stacking interaction between the adenine base and the 2′-base.

Conformation of the Sugar Ring, Sugar–Phosphate Backbone, and Glycosidic Bond

In order to gain some insight into the details of the geometry and local conformational changes of these molecules, in particular A^U_U and A^G_G, the spin–spin coupling constants (J_{H-H}, J_{C-P}, J_{H-P}) were analyzed.

Conformation of the Ribose Ring. (*A*) *Adenosine Sugar Ring.* Information concerning the conformation of the adenosine ribose ring in A^U_U and A^G_G can be obtained from the vicinal spin coupling constants $^3J_{H1'-H2'}$, $^3J_{H2'-H3'}$, and $^3J_{H3'-H4'}$. The J values measured for these nucleotides are collected in Table VI. The standard computer simulation–iteration program LAME (Emsley et al., 1965) was employed to obtained accurate values for spin–spin coupling and chemical shifts. Simulation of the H2′ and H3′ proton resonances yielded accurate $^3J_{H-P}$ values ($J_{H2'-P2'}$, $J_{H2'-P3'}$, and $J_{H3'-P3'}$) giving information about the conformation of the C2′–O2′ and C3′–O3′ bonds (vide infra).

The values of $J_{H2'-H3'}$ and the sum $J_{H1'-H2'} + J_{H3'-H4'}$ are essentially constant in these molecules (Table VI) and other branched trinucleotides (data not shown), which is a necessary condition for the analysis of ribose conformations in terms of a rapid equilibrium between two puckered ring forms (Altona & Sundaralingam, 1972, 1973). The values of $J_{H1'-H2'}$ (6–7 Hz) and $J_{H3'-H4'}$ (2–3 Hz) varied slightly over the series. The measured values of $J_{H2'-H3'}$ (5.1 ± 0.2 Hz) and the sum ($J_{H1'-H2'} + J_{H3'-H4'}$, 9.0 ± 0.2 Hz) are, within experimental error, the same as those observed for purine 3′-nucleotides (5.2 ± 0.1 and 9.3 ± 0.2 Hz, respectively) and purine 2′-nucleotides, (5.2 ± 0.1 and 9.1 ± 0.2 Hz, respectively) (Davies & Danyluk, 1975; Ezra et al., 1976; Lee et al., 1976). These values are consistent with the furanose in an equilibrium between the C2′-endo (^{2}E, or S form) and C3′-endo (^{3}E, or N form) conformations (Altona & Sundaralingam, 1972, 1973; Figure 4). To a good approximation the equilibrium constant (K_{eq}) can be calculated directly from the observed $J_{H1'-H2'}$ and $J_{H3'-H4'}$ values, which are the weighted averages of J values of the pure N and S conformers, according to eq 1 (Davies & Danyluk, 1974)

$$K_{eq} = X_S/X_N = J_{H1'-H2'}/J_{H3'-H4'} \quad (1)$$

$$X_S + X_N = 1 \quad (2)$$

where X_N is the fraction of the N conformer and X_S is the fraction of the S conformer. From eq 1 and 2, eq 3 and 4 may be derived.

$$X_S = J_{H1'-H2'}/(J_{H1'-H2'} + J_{H3'-H4'}) \quad (3)$$

$$X_N = J_{H3'-H4'}/(J_{H1'-H2'} + J_{H3'-H4'}) \quad (4)$$

With these equations the percentage of S population (% ^{2}E = $X_S \times 100$) was computed for the branched furanose rings. The data, summarized in Table VII, show some very interesting trends. The branched sugar rings show a definite preference for the ^{2}E pucker with the preference being detectably greatest for those sugar rings linked to pyrimidine residues through the 2′–5′ phosphodiester bond. Thus, the percentage of the ^{2}E conformer of the adenosine sugar ring in A^U_U, A^T_T, A^U_G, and A^C_G is about 80% and decreases to about 70% in A^G_G, A^G_C, and A^G_U. This trend suggests a correlation between the 2′-residue sequence and the central ribose conformational equilibrium.

(*B*) *Sugar Rings of the 2′- and 3′-Residues.* The measured values of $J_{H2'-H3'}$ (5.2 ± 0.2 Hz) and the sum ($J_{H1'-H2'} + J_{H3'-H4'}$, 9.5 ± 0.2 Hz) for the 2′- and 3′-residues of the branched trinucleotides are, within experimental error, the same as those observed for 5′-nucleotides (5′pX) (Ezra et al., 1977), and these values are consistent with the furanose being in an equilibrium between ^{2}E and ^{3}E conformers. The % ^{2}E values of the 2′- and 3′-residues are collected in Table VII. The data show that in all cases the 2′-residues have a greater ^{3}E conformer population (smaller $J_{H1'-H2'}$ values) than the 3′-residues: i.e., (i) the furanose of 2′-pyrimidines shows significant, sometimes exclusive, preference (70–100%) for the

[2] The CD spectra of A^U_U showed a blue shift of the long-wavelength peak, λ_+ (from 276 to 271 nm), with increasing temperature (22–72 °C). A similar shift has been observed for A^pU (Doornbos et al., 1981). In contrast, the λ_+ peak in the CD spectrum of A_pU does not shift in this temperature range.

Table VI: ^{1}H NMR Coupling Constants[a] for Branched Trinucleotides and Dinucleoside Monophosphates in D_2O[b]

nucleotide		temp (°C)	1′2′	2′3′	3′4′	4′5′	4′5″	2′P2′	2′P3′	3′P3′	4′P	5′P	5″P
A^U_U	A	22	7.3	5.0	1.8	2.4	2.5	9.6	2.2	6.8			
	−2′pU		3.0	5.1	6.5	2.0[c]	3.0				2.5[c]	4.6[c]	3.3
	−3′pU		5.5	5.4	4.0	2.6	3.9				[c]	4.7	5.8
A^U_U	A	50	7.2	5.2	1.9	2.7	2.6	9.4	2.0	7.1			
	−2′pU		3.4	5.1	5.9	2.4[c]	3.2[c]				[c]	5.1[c]	3.5[c]
	−3′pU		[c]	[c]	[c]	2.8	4.0				[c]	5.0	6.0
A^U_U	A	70	7.0	5.1	2.1	2.5	3.1	9.2	1.9	7.0			
	−2′pU		3.7	5.1	5.5	2.7[c]	3.3[c]				[c]	5.1[c]	3.7[c]
	−3′pU		[c]	[c]	[c]	2.9	4.0				[c]	5.1	6.1
A^G_G	A	25	6.3	5.0	2.6	2.9	2.5	9.2	1.6	7.3			
	−2′pG		5.0	5.0	4.4	2.9	2.9				2.5	4.7	3.8
	−3′pG		5.5	5.2	4.0	3.0	4.4				1.1	5.0	5.5
A^G_G	A	50	6.3	5.0	2.5	2.9[d]	2.9[d]	9.2	1.6	7.5			
	−2′pG		5.3	5.1	4.2	3.1	3.3				2.3	5.2	4.4
	−3′pG		5.7	5.4	4.0	3.2	4.5				1.3	5.0	5.6
A^G_G	A	70	6.4	5.0	2.6	3.0[d]	3.0[d]	9.2	1.6	7.5			
	−2′pG		5.6	5.2	3.8	3.6	3.5				2.0	5.6	4.8
	−3′pG		6.0	5.3	3.9	3.4	4.7				1.2	5.2	5.7
A_pU[e]	A	20	3.8	5.2	5.4	3.0	2.1			8.4			
	−pU		3.5	5.2	5.6	2.2	2.3				2.0	3.5	3.7
A^pU[f]	A	38	6.6	5.1	2.7	2.4	3.1	9.1					
	−pU		3.0	5.0	6.5	2.0	2.6				2.5	3.5	3.2
A^pU[f]	A	78	6.2	5.2	3.2	2.7	3.7	8.8					
	−pU		3.5	5.2	6.0	2.5	2.6				2.3	4.0	4.2
A_pG[e]	A	20	4.6	4.9	4.7	2.4	3.4			8.2			
	−pG		4.4	5.2	4.8	2.8	3.6				2.2	4.0	4.0
A_pG[e]	A	80	5.4	5.2	3.8	2.9	3.8			8.0			
	−pG		4.9	5.4	4.6	2.6	4.7				1.0	4.8	5.3
A^pG	A	25	4.6	5.0	4.8	2.5	3.5	9.0					
	−pG		4.5	5.1	4.9	2.3	2.7				2.4	3.6	3.8
A^pG	A	70	5.3	5.2	4.2	2.8	4.0	8.7					
	−pG		5.0	5.2	4.3	3.3	2.9				2.1	4.7	4.5

[a] Coupling constants accurate to ±0.1–0.3 Hz. [b] Solution conditions are the same as in Table IV. [c] Overlapping of signals precluded exact determination. [d] H5′ and H5″ are magnetic equivalents at this temperature. [e] Data from Danyluk et al. (1976, 1977). [f] Data from Doornbos et al. (1981).

^{3}E pucker (e.g., the 2′-cytidines in A^C_C and A^C_G);[3] (ii) 3′-pyrimidines and 2′-guanosines have nearly equal ^{2}E and ^{3}E rotamer populations (e.g., A^G_C and A^G_U); (iii) the ribose conformational equilibrium of 3′-guanosines shows a slight preference (60–65%) for the ^{2}E pucker (e.g., the 3′-guanosines in A^G_G, A^C_G, and A^U_G).

(*C*) *Trimerization Effect on Coupling Constant.* Comparison of the conformations of the sugar rings of the 2′–5′- and 3′–5′-linked dimers A^pU, A_pU, A^pG, and A_pG with those of A^U_U and A^G_G further supported our hypothesis about the base–base stacking interaction in these molecules. The % ^{2}E values of these dimers at various temperatures are collected in Table VII. The $^3J_{H-H}$ and $^3J_{H-P}$ values of A_pU, A^pU, and A_pG, presented in Table VI, were obtained from the studies of Ezra et al. (1976), Lee et al. (1976), and Doornbos et al. (1981) (pD 7.0, 10–30 nM). The conformation of the adenosine sugar moiety in A^pU and A_pU is strongly influenced by the position of the phosphodiester linkage. The preferred sugar conformation of A^pU is S^pN, while that of A_pU is N_pN. The S^pN puckering mode appears to be a common feature of 2′–5′-linked purine–pyrimidine dimers and has been previously commented upon for A^pU, both in solution (Doornbos et al., 1981) and in the crystal structure (Shefter et al., 1964, 1969), as well as for G^pU (Dhingra et al., 1978) and A^pC (Kondo et al., 1970). As previously discussed, the A^pU constitutive fragment of A^U_U also prefers an S^pN puckering, and therefore, the conformation of the furanoses in the dinucleoside monophosphate A^pU is preserved upon trimerization. The S^pN puckering appears to promote base stacking between the adenine and 2′-uracil chromophores in both A^pU and A^U_U.

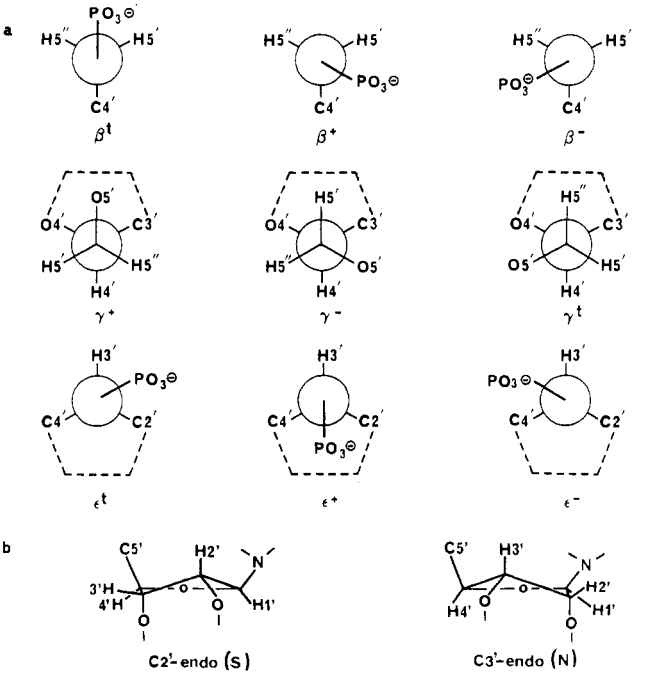

FIGURE 4: (a) Classical staggered rotamers for C4′–C5′, C5′–O5′, and C3′–O3′ bonds. (b) C2′-endo (S) and C3′-endo (N) conformations of the furanose ring.

The effect of trimerization on the ^{2}E–^{3}E population distribution of the uridine residue of A_pU is worth noting. The ribose moiety of the 3′-uridine undergoes a decisive change to increased ^{2}E (S) pucker upon trimerization; i.e., % ^{2}E

[3] In the case of aA^U_U, both 2′- and 3′-furanoses have the same ^{2}E rotamer populations ($^3J_{H1'-H2'}$ = 4.2; % ^{2}E = ca. 44). The ^{2}E, ^{3}E rotamer populations of the 2′- and 3′-furanoses of A^T_T were not determined.

Table VII: Population Distribution of Conformers in Branched Trinucleotides and Dinucleoside Monophosphates

	A^{U}_{U}			$A^{p}U$		A_pU		$^{p}U^{g}$	A^{G}_{G}			$A^{p}G$		A_pG		^{p}G
	A	2′U	3′U	A	U	A	U	U	A	2′G	3′G	A	G	A	G	G
% ^2E[a]	80	32	58	71	32	41	38	53	71	53	58	49	48	49	48	64
% γ^+[a]	87	86[b]	70	80	90	85	91	85	81	77	61	75	86	77	71	67
% β^t[c]		85[b]	72		91		88	78		82	72		87		84	74
% β^t[d]		83	75		86[e]		85			85	77		85		84	
temp (°C)		22		38		20		20		25		25		20		20
% ^2E[a]	77	40	*b*	66	37				71	60	61	56	54	59	52	
% γ^+[a]	79	75[b]	66	71	85				75	64	54	67	73	68	62	
% β^t[c]		80[b]	69		83					73	70		78		74	
% β^t[d]		80	72		74[f]											
temp (°C)		70		78						70		70		80		

	A^{U}_{G}			A^{C}_{C}			A^{C}_{G}			G^{U}_{U}			A^{G}_{U}			A^{G}_{C}		
	A	U	G	A	2′C	3′C	A	C	G	G	2′U	3′U	A	G	U	A	G	C
% ^2E (22 °C)	82	24	57	81	0	47	81	11	65	78	25	46	64	52	46	71	46	46

[a] % $^2E = J_{H1'-2'}/(J_{H1'-H2'} + J_{H3'-H4'})$; % $\gamma^+ = (13.3 - J_{H4'-H5'} - J_{H4'-H5''})/9.7$. [b] Overlapping signals precluded exact determination. [c] % $\beta^t = (25.38 - J_{H5'-P5'} - J_{H5'-P5''})/20.63$. [d] % $\beta^t = (J_{C4'-P} - 0.73)/10.27$. [e] 20 °C. [f] 70 °C. [g] % γ^+ and % β^t of uridine 5′-(methyl phosphate) are 79% and 78%, respectively (Davies, 1978).

changes from 38% (A_pU) to 58% (A^{U}_{U}), a value quite similar to that of uridine 5′-monophosphate (% ^2E = 53%). The same effect is observed when A_pU is heated to 70 °C (the uridine % ^2E changes from 38% to 47%), at which temperature the population of stacked species in this dimer is very small (Ezra et al., 1977). Since it is the N_pN puckering mode that promotes stacking in 3′-5′-linked dinucleotides, our results suggest that the 3′-uridine residue of A^{U}_{U} is not base stacked, as concluded from the CD and ^1H chemical shift studies. These results clearly illustrate the interrelation between base stacking and ribose conformation.

Comparison of the sugar conformations of $A^{p}G$, A_pG, and A^{G}_{G} leads to the same conclusions (Table VII). The 3′-guanosine residue of A_pG undergoes a substantial shift to the ^2E form upon trimerization (% ^2E changes from 48% to 58%). A similar effect, but less pronounced, is observed when A_pG is heated from 20 to 80 °C (% ^2E increases from 48% to 52%). The effect of trimerization on the population distribution of ribose conformers in $A^{p}G$ follows the pattern observed for $A^{p}U$, i.e., a shift to the ^2E form for the adenosine sugar and little change in the conformer population of the 2′-guanosine residue. Again, these results are in accord with the existence of an extended conformation for the 3′-guanosine residue and a base stack between the adenine and 2′-guanine bases.[4]

Sugar–Phosphate Backbone. (A) C3′–O3′ (ε) and C2′–O2′ (ε′) Bonds. Simple steric considerations show that branched trinucleotides could be analyzed in terms of a three-state equilibrium about the C3′–O3′ and C2′–O2′ bonds. Thus, one could envisage the possibility of three distinct conformers differing only in the position in which the P3′–O3′ or P2′–O2′ bonds are placed in both gauche regions with respect to the C3′–H3′ and C2′–H2′ bonds, respectively: e.g., (i) ϵ^t, ϵ'^t, (ii) ϵ^-, ϵ'^t, and (iii) ϵ^-, ϵ'^- (Figure 5). A fourth conformer in which both phosphodiester groups are directed toward each other, i.e., (iv) ϵ^t, ϵ'^-, can be excluded for steric reasons and electrostatic repulsion of the charged phosphodiester groups. We also excluded from this equilibrium molecules containing ϵ^+ rotamers (Figure 4), as the ϵ^+ region appears to be "forbidden" in 3′–5′-linked nucleotides (Sundaranlingam, 1969; Altona et al., 1974; Alderfer & Ts'o, 1977). Similarly, the existence of the ϵ'^+ conformation in the 2′–5′-linked residue is very unlikely. A space-filling CPK model of A^{U}_{U} indicates considerable difficulty in achieving base–base stacking interactions (adenine–2′-uracil) with the C2′–O2′ bond in the ϵ'^+ conformation. Furthermore, there is steric interference upon conversion of the ϵ'^t and ϵ'^- rotamers to the ϵ'^+ rotamer. Thus, the C3′–O3′ and C2′–O2′ bonds were analyzed, individually, in terms of an equilibrium between the two allowed conformers, ϵ^t, ϵ^- and ϵ'^t, ϵ'^-, respectively. This analysis is in perfect agreement with all the subsequent NMR data.

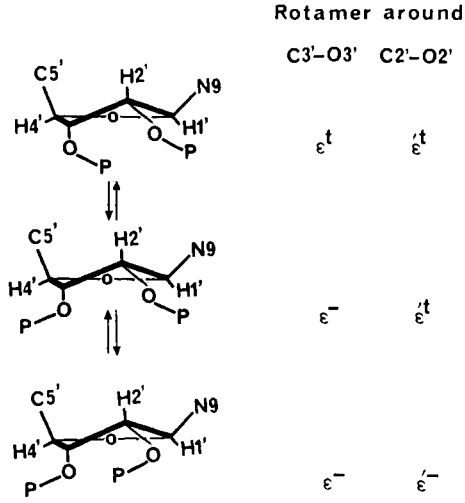

FIGURE 5: C3′–O3′ and C2′–O2′ bond conformations of branched trinucleotides.

The conformation of the C3′–O3′ bond in A^{U}_{U} and A^{G}_{G} was determined from the value of $^3J_{HCOP}$ between the phosphate $^{31}P3'$ and the vicinal H3′ and from $^3J_{CCOP}$ magnitudes observed between $^{31}P3'$ and both C2′ and C4′ (Figure 4). On the basis of a large data set of coupling constants $^3J_{CCOP}$ and $^3J_{HCOP}$ of 3′–5′-linked RNA fragments, Lankhorst et al. (1984) derived Karplus relationships between torsion angles θ and θ' and $^3J_{HP}$ and $^3J_{CP}$ values:

$$^3J_{CCOP} = 6.9 \cos^2 \theta - 3.4 \cos \theta + 0.7 \quad (5)$$

$$^3J_{HCOP} = 15.3 \cos^2 \theta' - 6.1 \cos \theta' + 1.6 \quad (6)$$

Here

$$\theta = \text{torsion angle C4′–C3′–O3′–P3′} \quad (7)$$

$$\theta' = \text{torsion angle H3′–C3′–O3′–P3′} \quad (8)$$

$$\theta = 240 - \theta'$$

[4] It should be noted that while $A^{p}U$ displays a $S^{p}N$ stacked state, $A^{p}G$ does not. The sugar residues of $A^{p}G$ have nearly equal populations of ^2E and ^3E conformers, a feature also observed in the dimer $A^{p}A$ by Doornbos et al. (1981) which, they concluded, is due to a mixed mode of stacking (i.e., $N^{p}N$, $N^{p}S$, $S^{p}N$, and $N^{p}N$).

Table VIII: ^{13}C–^{31}P Coupling Constants[a] for Branched Trinucleotides (X^{Y}_{Z}) and Dinucleoside Monophosphates ($X^{p}Y$, $X_{p}Z$) in D_2O[b]

		X						Y	Z
nucleotide	temp (°C)	C1′–P2′	C2′–P2′	C3′–P2′	C2′–P3′	C3′–P3′	C4′–P3′	C4′–P2′	C4′–P3′
A^{U}_{U}	22	7.8	5.0	2.5	7.3	5.4	*b*	9.3	8.4
A^{U}_{U}	60	6.8	5.0	3.2	6.9	5.2	~1.0	8.9	8.1
$A^{p}U$	20	8.5	5.0	1.8				9.6	
$A^{p}U$	60	8.3	5.2	2.7				8.3	
$A_{p}U$	22				4.2	4.9	5.3		9.5
$A_{p}U$	60				5.4	4.5	4.2		8.6
A^{G}_{G}	22	6.5	5.1	~3[c]	6.2	~5[c]	*b*	9.5	8.6
$A^{p}G$	21	6.9	5.6	3.1				9.5	
$A_{p}G$	22				4.2	5.7	4.4		9.4
A^{T}_{T}	22	7.7	~5[c]	~3[c]	~7[c]	~5[c]	*b*	9.5	8.6
aA^{U}_{U}	23	5.5	5.3	3.5	7.7	4.7	2.4	8.4	8.5
A^{C}_{C}	22	7.5	5.1	2.5	7.1	5.6	*b*	9.6	8.0
G^{U}_{U}	24	7.1	5.1	2.8	7.0	4.9	*b*	9.3	8.4

[a] ±0.2 Hz. [b] Unresolved doublet due to small coupling constant (0–0.9 Hz). [c] Bad signal/noise prohibited exact determination.

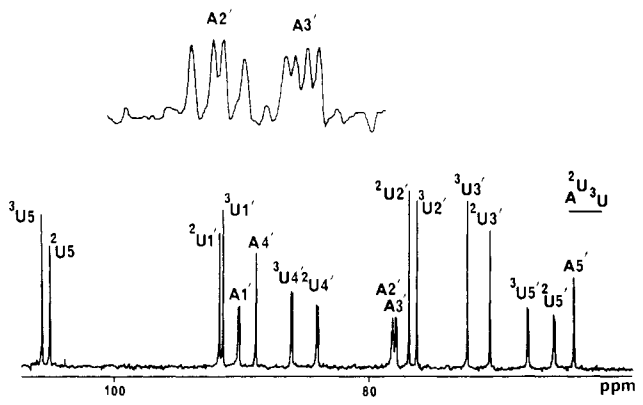

FIGURE 6: ^{13}C NMR (75.4 MHz) spectrum of A^{U}_{U} in D_2O (pD 7–8).

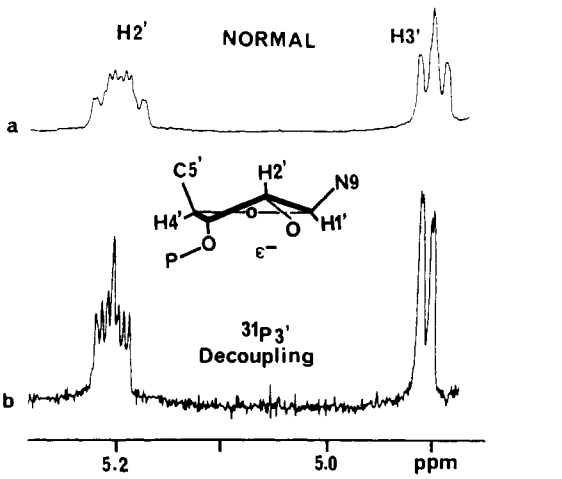

FIGURE 7: The W conformation of the H2′–C2′–C3′–O3′–P3′ fragment. (a) Adenosine H2′ and H3′ resonances (400 MHz); (b) as in (a) except an irradiation power was applied at the P3′ resonance.

These authors concluded that the conformation of the C3′–O3′ bond in ribosyl dinucleoside monophosphates is best considered as a rapid equilibrium between ϵ^{t} and ϵ^{-} conformers for which they calculated torsion angles θ of 214–226° (average 219°) and 277°, respectively. Observed $^{3}J_{CCOP}$ and $^{3}J_{HP}$ values represent time-averaged coupling constants of the relative populations of these two conformers. Using eq 5–8, one determines $^{3}J_{C4'-P3'}$ values of 7.4 (J_{CP^t}) and 0.5 Hz (J_{CP^-}) and $^{3}J_{H3'-P3'}$ of 9.2 (J_{HP^t}) and 6.9 Hz (J_{HP^-}), for the pure ϵ^{t} (θ = 219°) and ϵ^{-} (θ = 275°) conformers, respectively.

The ^{13}C–^{31}P coupling constants of A^{U}_{U}, $A^{p}U$, $A_{p}U$, A^{G}_{G}, $A^{p}G$, $A_{p}G$, and other branched trinucleotides are shown in Table VIII. The ^{13}C NMR spectrum of A^{U}_{U} (95–60 ppm region) is shown in Figure 6. As expected, both C3′ and C2′ consisted of two sets of doublets due to geminal and vicinal couplings to $^{31}P2'$ and $^{31}P3'$. The geminal couplings were easily identified since their values are characteristically 5.0 ± 0.5 Hz and are invariant with temperature (Lankhorst et al., 1984). A striking feature in the ^{13}C NMR spectrum of A^{U}_{U} (and other branched trinucleotides we analyzed) is the very small $^{3}J_{C4'-P3'}$ value of the adenosine residue. The C4′ signal appeared as a "singlet" and was just resolved at 60 °C ($^{3}J_{C4'-P3'}$ = 1.0 Hz). Because the spectral resolution we used allowed us to resolve doublets with J_{CP} values greater than or equal to 1.0 Hz, we estimate the value of this coupling to be in the range of 0–0.9 Hz at room temperature. The value of $^{3}J_{H3'-P3'}$ observed was 6.8 Hz. These $^{3}J_{C4'-P3'}$ and $^{3}J_{H3'-P3'}$ values are almost identical with those expected for the pure ϵ^{-} rotamer (θ = 275°), 0.5 and 6.9 Hz, respectively, and indicate that this rotamer is exclusively populated at 22 °C. A further piece of information, namely, the observation of a long-range coupling $^{4}J_{H2'-P3'}$ of 2.2 Hz, confirmed unequivocally the high population of the ϵ^{-} rotamer. This four-bond coupling is only possible along a planar "W" path created when the adenosine sugar ring adopts the ^{2}E conformation and the P3′–O3′ bond is antiperiplanar to the C3′–C2′ bond (ϵ^{-} orientation; Figure 7). Studies by Hall and Malcom (1972a,b) and Donaldson and Hall (1972) indicate that the magnitude of $^{4}J_{HP}$ coupling in W-oriented H–C–C–O–P fragments of cyclic phosphate esters exhibits a maximum value of about 2.7 Hz and suggests a reduction to about 0 Hz when this relationship is absent. To the best of our knowledge the magnitude of $^{4}J_{H2'-P3'}$ we measured for A^{U}_{U} (2.2 Hz) is the largest so far reported for a nucleotide fragment, and its large magnitude reflects the high conformational purity about the C3′–O3′ bond (ϵ^{-} rotamer) indicated by the observed $^{3}J_{C4'-P3'}$ and $^{3}J_{H3'-P3'}$ values. Since it is only the ϵ^{t} rotamer that allows base stacking in the dimer $A_{p}U$ and other 3′–5′-linked RNA fragments (Sarma et al., 1977), one can definitely conclude that the 3′-uridine residue of A^{U}_{U}, on average, does not participate in any base–base stacking with the adenine residue, in agreement with all previously discussed NMR and CD results.

One important suggestion, supported by theoretical potential energy calculations (Dhingra & Saran, 1982) and NMR studies (Lee et al., 1976; Ezra et al., 1977; Davies & Sadikot, 1983), is that the sugar-ring puckering equilibrium is related to the C3′–O3′ bond conformation. The N-type (^{3}E) ribose ring is exclusively associated with the ϵ^{t} conformer, whereas the S-type (^{2}E) ring is associated with the ϵ^{-} conformer. The following findings support this suggestion: (i) There is a large population of both ^{2}E (adenosine sugar, vide supra) and ϵ^{-} rotamers in A^{U}_{U}. Elevation of temperature from 22 to 75 °C

causes a small depopulation of both ^2E and ϵ^- conformers; the slight decrease in ϵ^- was monitored by the change in $^4J_{H2'-P3'}$ (2.2 to 1.9 Hz), $^3J_{C4'-P3'}$ (~0.5 to 1.0 Hz), $^3J_{C2'-P3'}$ (7.3 to 6.9 Hz), and $^3J_{H3'-P3'}$ (6.8 to 7.1 Hz) values. In contrast, no change in the population of these rotamers is observed in A^G_G ($^4J_{H2'-P3'}$ = 1.6 Hz and $^3J_{H1'-H2'}$ = 6.3 Hz, at 22 and 75 °C). (ii) A^G_G, A^G_U, and A^G_C, whose ^2E (adenosine) conformer populations are slightly smaller than those of A^U_U and A^U_G (vide supra), showed a correspondingly smaller ϵ^- rotamer population (smaller $^4J_{H2'-P3'}$ and higher $^3J_{H3'-P3'}$ values; Tables VI and VII).

With eq 5 and 7, the expected magnitudes of $^3J_{C4'-P3'} + {}^3J_{C2'-P3'}$ for the pure ϵ^- and ϵ^t rotamers are 9.9 (0.5 + 9.4 Hz; θ = 275°) and 8.8 Hz (7.4 + 1.4 Hz; θ = 219°), respectively. Because the C3′–O3′ bond adopts exclusively the ϵ^- orientation, we expected $J_{C4'-P3'} + J_{C2'-P3'}$ to be in the vicinity of 10 Hz. The observed $^3J_{C4'-P3'} + {}^3J_{C2'-P3'}$ sum for A^U_U (and other branched trinucleotides, Table VIII), however, was found to be about 7.8 Hz (~0.5 + 7.3 Hz, respectively). This low sum appears to be due to a significant decrease (2.1 Hz) in $^3J_{C2'-P3'}$. The smaller than expected $^3J_{C2'-P3'}$ value may be due to the electronegative 2′-substituent (2′-phosphate group) which is absent in 3′–5′ RNA fragments. Lankhorst et al. (1984) studied the change of substitution pattern of C2′ by comparing 3′,5′-cAMP with 2′-deoxy-3′,5′-cAMP and found that the 2′-oxygen substituent had no measurable effect on $^3J_{C2'-P3'}$. This is in apparent contradiction to our hypothesis. On the other hand, some authors (Alderfer & Ts'o, 1977; Rycyna & Alderfer, 1985) have observed that $^3J_{C4'-P} + {}^3J_{C2'-P}$ in many ribosyl dinucleoside monophosphates is 9.2 ± 0.2 Hz, whereas for deoxyribosyl dinucleoside monophosphates this sum is 1.0 Hz higher (10.2 ± 0.2 Hz), indicating a substituent electronegativity effect (C2′-*H* versus C2′-*OH*) on the magnitude of $J_{C2'-P}$.[5] Due to the charged nature of the 2′-phosphate substituent, one would expect the observed $J_{C2'-P3'}$ value to decrease, if the electronegative effect operates, in the order deoxyribosyl > ribosyl > branched ribosyl nucleotide fragments, as indicated by the experimental sums of 10.2 ± 0.2 Hz > 9.2 ± 0.2 Hz > 7.5 ± 0.5 Hz, respectively.

Overlapping of the H2′ and H3′ resonances of the arabinose sugar in aA^U_U prohibited the determination of $J_{H3'-P3'}$. The magnitudes of $^3J_{C4'-P3'}$ (2.4 Hz) and $^3J_{C2'-P3'}$ (7.7 Hz), however, suggest that the C3′–O3′ bond is primarily in the ϵ^- conformation, but with some population in the ϵ^t conformation as well. The exciton splitting observed in its CD spectrum must therefore arise from the stacking of the adenine and the 2′-uracil bases. Again, we observed that the sum $^3J_{C2'-P3'} + {}^3J_{C4'-P3'}$ of this compound (10.1 Hz) was smaller than that of two 3′–5′-linked arabinonucleotides we measured, namely, aU_paU (11.0 Hz) and aA_paA (10.8 Hz) (Damha et al., 1987).

Analysis of the three-bond coupling constant $^3J_{C1'-P2'}$ and $^3J_{C3'-P2'}$ yielded information about the conformation of the C2′–O2′ bond. The large $^3J_{C1'-P2'}$ and small $^3J_{C3'-P2'}$ values (Table VIII), and the absence of a detectable $^4J_{HP}$ between ^{31}P2′ and either H1′ or H3′, for A^U_U, A^pU, A^G_G, and A^pG are in agreement with the existence of a preferred ^2E (adenosine) sugar conformation and the P–O2′ bond predominantly antiperiplanar to the C2′–C1′ bond, i.e., high population of ϵ^- rotamer.

At this stage a comparison of the sums $^3J_{C1'-P2'} + {}^3J_{C3'-P2'}$ of A^pU and A^U_U appears appropriate. At 60 °C these sums were 11.0 and 10.0 Hz for A^pU and A^U_U, respectively, indicating an electronegativity effect on $J_{C3'-P2'}$ by the 3′-phosphate group. At 22 °C, however, these sums were identical for both compounds (10.3 Hz). Due to the lack of ^{13}C NMR data on 2′–5′-linked nucleotides in the literature,[6] no further comparison of the magnitude of these sums was possible.

(B) C4′–C5′ (γ) and C5′–O5′ (β) Bonds. The conformational preference about the C4′–C5′ bond is best monitored by means of the vicinal ^{1}H–^{1}H couplings $J_{H4'-H5'}$ and $J_{H4'-H5''}$, whereas that about the C5′–O5′ bond is monitored by the vicinal ^{1}H–^{31}P and ^{13}C–^{31}P couplings $^3J_{H5'-P}$, $^3J_{H5''-P}$, and $^3J_{C4'-P}$ (Figure 4). The magnitudes of these coupling constants for A^G_G and A^U_U and the corresponding 2′–5′ and 3′–5′ dinucleoside monophosphate dimers are given in Tables VI and VIII. The relative populations of the β^t rotamer of these nucleotides were calculated from the simple sum rules (eq 9 and 10), derived from the Karplus equations (eq 5 and 6).[7]

$$\% \beta^t = 100(J_{C4'-P} - 0.73)/10.27 \quad (9)$$

$$\% \beta^t = 100[25.4 - (J_{H5'-P} + J_{H5''-P})]/20.6 \quad (10)$$

The population of the γ^+ rotamer was calculated from the sum rule suggested by Altona (1983):

$$\% \gamma^+ = 100[13.3 - (J_{H4'-H5'} + J_{H4'-H5''})]/9.7 \quad (11)$$

Individual γ^-, γ^t, β^+, and β^- rotamer populations can be calculated by using similar equations. For the purpose of our studies, however, it was only necessary to calculate those for the γ^+ and β^t rotamers.

The data in Table VII clearly demonstrate that γ^+ and β^t of all 2′- and 3′-residues in branched trinucleotides and dinucleoside monophosphates are highly populated. Comparison of the γ^+ and β^t rotamer populations of the 2′- and 3′-residues in A^U_U[8] and A^G_G demonstrates, however, a larger conformational purity in the former, i.e., larger $^3J_{C4'-P}$ (Table VIII) and smaller $J_{H4'-H5''} + J_{H4'-H5''}$ and $J_{H5'-P} + J_{H5''-P}$ sums (Table VI).

An important stereochemical consequence of the 2′- and 3′-residues existing in the γ^+, β^t conformation is that the atoms

[5] Davis and Sadikot (1982) have estimated from $^3J_{C1'-P2'}$ couplings in cyclic 2′,3′-mononucleotides that substitution of a proton located on the coupled ^{13}C1′ by a nitrogen (N1′ of base) causes an increase (a reverse effect) of 1.1 Hz in the vicinal $^3J_{C1'-P2'}$.

[6] To the best of our knowledge, there has been only one report in the literature on the ^{13}C NMR data of a 2′–5′-linked dimer, namely, A^pA. The sum $J_{C1'-P2'} + J_{C3'-P2'}$ of this nucleotide was 4.9 (37 °C) and 8.9 Hz (80 °C) (Schleich et al., 1976).

[7] Equation 9 was derived as follows: on the assumption that each of the three β bond staggered conformers is populated (Figure 4) and that interconversion between rotamers is rapid on the NMR time scale, the relative population of β^t (X^t) may be estimated from the observed $^3J_{C4'-P}$ and from knowledge of $^3J_{CP}$ of each conformer, namely, $^3J_{CP}{}^+(60°)$, $^3J_{CP}{}^-(-60°)$, and $^3J_{CP}{}^t(180°)$ calculated from the Karplus equation (eq 5) as 0.73, 0.73, and 11.00 Hz, respectively. Here

$$X^t + X^- + X^+ = 1$$

and

$$J_{C4'-P} = 11.0X^t + 0.73(X^- + X^+)$$

Combining and rearranging gives

$$x^t = (J_{C4'-P} - 0.73)/11.00 - 0.73$$

or

$$\% \beta^t = 100X^t = 100(J_{C4'-P} - 0.73)/10.27 \quad (9)$$

Equation 10 was derived in a similar fashion by using $^3J_{HP}$ values of 23.0 and 2.4 Hz for the trans (180°) and gauche (60°) couplings, respectively, calculated from the Karplus equation (eq 6).

[8] Overlapping of signals prohibited the exact determination of $J_{H4'-H5'}$ and $J_{H5'-P}$ of the 2′-uridine residue in A^U_U. The magnitudes of these couplings, % γ^+ and % β^t, reported in Tables VI and VIII are approximate values.

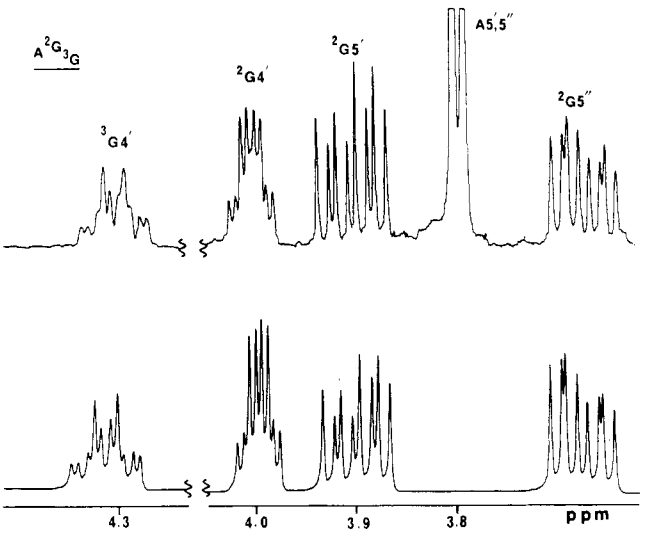

FIGURE 8: ^{1}H NMR (300 MHz) spectrum of A^G_G between 4.5 and 3.5 ppm (50 °C, top) and the corresponding simulation (bottom).

H4′, C4′, C5′, O5′, and P5′ are in the same plane. The W-type geometry of the H4′–C4′–C5′–O5′–P5′ fragment is favorable for a $^4J_{H4'-P5'}$ coupling constant. One therefore expects a strong correlation to exist between high populations of γ^+ and β^t and the magnitude of $^4J_{H4'-P5'}$. Indeed, Sarma et al. (1973) have obtained linear correlations for several mononucleotides and estimated a value of 2.8 Hz for a pure γ^+, β^t combination. Values of 3.0–3.7 Hz have been observed (Altona, 1982). The $^4J_{H4'-P5'}$ couplings for the 2′- and 3′-residues in A^U_U and A^G_G were unambiguously identified by ^{1}H–^{31}P heterodecoupling and their magnitudes determined by careful computer simulation of the H4′ resonances (Figure 8). The magnitude of $^4J_{H4'-P5'}$ was found to be 2.5 and 1.1 Hz for the 2′- and 3′-guanosine residues (25 °C), respectively, indicating again larger populations of γ^+ and β^t for the 2′-guanosine residue (Table VII). Overlapping of signals prohibited the accurate determination of $^4J_{H4'-P5'}$ for both the 2′- and 3′-residues in A^U_U. The significantly increased γ^+ and β^t populations in the 2′-residue compared to the 3′-residue (Table VII) are ascribed to the predominating presence of a conformationally ordered stacked state, which is apparently only possible when the C4′–C5′ and C5′–O5′ bonds assume the γ^+ and β^t conformation.

The effect of trimerization on the γ^+ and β^t populations of the uridine and guanosine residues in A_pG and A_pU, i.e., a decrease of 10–20% in the population of both rotamers, further indicates that the 3′-residues in A^G_G and A^U_U are not based stacked. Interestingly, the γ^+ and β^t populations of the 3′-residues of A^G_G and A^U_U were found to be smaller that those of the uridine and guanosine 5′-monophosphate monomers. On the other hand, the γ^+ and β^t populations of the uridine and guanosine residues in A^pU and A^pG did not change significantly upon trimerization.

The effect of temperature on the populations of γ^+ and β^t of the 2′-residues (A^U_U and A^G_G) was significantly more profound than on those of the 3′-residues. The large decrease in the γ^+ and β^t populations of the 2′-residue with increasing temperature is ascribed to destacking of the molecule.

Glycosidic Bond Conformation of the Adenine Base. In CPK space-filling models, one observes that the ribose proton closest to H8 is H1′ if the conformation is syn and H2′ or H3′ if it is anti. This motivated us to measure the nuclear Overhauser enhancements (NOE) of H8 upon the saturation of the H1′, H2′, and H3′ protons, a procedure first exploited by Hart and Davies (1969a,b) and which was soon used by others (Son et al., 1972) in the field. The magnitude of the Overhauser effect depends on the inverse sixth power of internuclear distance so that relative values of NOE yield an estimate of various internuclear distances. The NOE can be described quantitatively as the fractional enhancement of the H8 resonance, f_8(H), when the resonances of H1′, H2′, and H3′ are saturated:

$$f_8(\mathrm{H}) = (\text{area of H8 when H is saturated} - \text{equilibrium area of H8})/\text{equilibrium area of H8}$$

The observed NOE of the H8 resonance, f_8(H), would be the time-averaged value of contributions from the syn and anti conformers. In A^U_U, the percentage of intensity enhancement of H8 when H1′ was irradiated was found to be 11%. Also, the percentage of H8 enhancement upon irradiation of H2′ and H3′ was found to be 10% and 3%, respectively. This result suggests that the adenine base is primarily in the syn conformation but with some population in the anti conformation as well. A given enhancement by H1′ is more significant (reflects more syn conformation) than the same degree of enhancement by H2′ or H3′ (which reflects anti conformation) because the minimum possible distance to H1′ is greater than for H2′ or H3′ regardless of the sugar puckering (^{2}E and ^{3}E; Son et al., 1972). Equal f_8(H1′) and f_8(H2′) enhancements therefore reflect more syn conformation. The f_8(H1′), f_8(H2′), and f_8(H3′) enhancements in A^G_G were found to be 5%, 1%, and 0%, respectively, indicating that the adenine base also favors the syn conformation about the glycosidic bond. These findings correlate well with normal purine nucleosides (DMSO-d_6 solutions) and nucleotides (D_2O), which exhibit predominantly syn conformations (Davies, 1978).

It is interesting that the relative populations of both the syn (glycosidic bond) and γ^+ (C4′–C5′ bond) conformers of the adenosine residue are highly populated in A^U_U and A^G_G (Table VII). This was not expected since the bulky base ring in the syn conformation is in close proximity to the O5′ atom in the γ^+ conformation. In fact, a decrease in γ^+ in going from anti type to syn type for both purine and pyrimidine derivatives has already been commented upon (Davies, 1978). It appears more likely that the adenine base in A^U_U and A^G_G is engaged in an intramolecular hydrogen bond O5′–H···N3. The syn, γ^+ combination has previously been observed for purine nucleosides in the solid state (exhibiting such intramolecular hydrogen bonding; Rao & Sundaralingam, 1970) and for 8-bromoadenosine in solution (Sarma et al., 1974).

Conclusions

In the present work it was shown that the conformation of branched trinucleoside diphosphates is scarcely influenced by the nature of the nucleoside residues. It is concluded from CD and NMR studies that the branched nucleoside base (adenine or guanine) stacks exclusively with the purine or pyrimidine base of the 2′-residue. This property was also observed in the trinucleotides aA^U_U and A^T_T.

A conformational model of A^U_U is presented in Figure 9. This model is a qualitative representation that incorporates structural details, of the most populated conformer, obtained from the CD and NMR analyses. The following conformational features are particularly evident in this model: (i) the adenine and 2′-uracil bases form a syn–anti (glycosidic bond), C2′-endo–C3′-endo (ribose conformation) stack; (ii) both phosphodiester linkages have the ϵ^- orientation; (iii) the W paths are H2′–C2′–C3′–O3′–P3′ (adenosine) and H4′–C4′–C5′–O5′–P2′ (2′-uridine); (iv) the extended (nonstacked) form of the 3′-uridine residue is present.

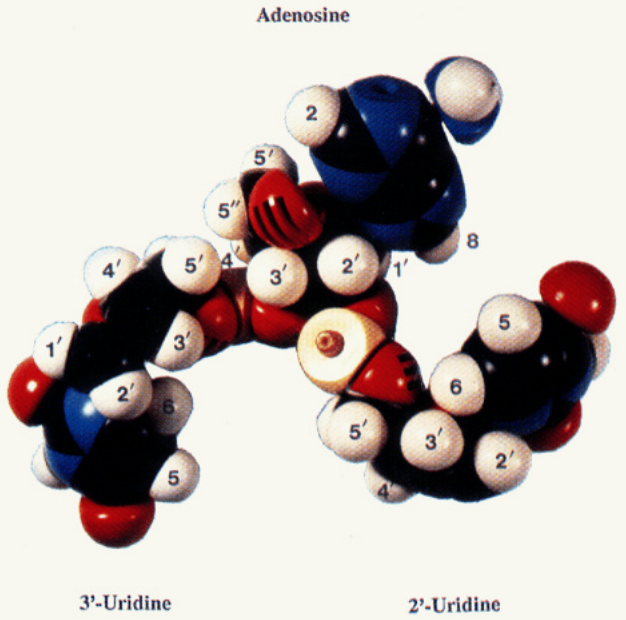

FIGURE 9: CPK molecular model of A^{U}_{U}. The 2′- and 3′-hydroxyl groups of the uridine residues have been removed for the sake of clarity.

The hypochromicity values of the naturally occurring trinucleotides A^{G}_{U} and A^{G}_{C} suggested that these nucleotides exhibited little base–base interaction. The nonconservative CD spectra of these nucleotides and the similarity of the NMR properties of these nucleotides to those of A^{G}_{G} and $A^{p}G$ indicate, however, that the adenine and guanine bases are base stacked. The interpretation of chemical shifts and coupling constants in terms of molecular geometry and conformation is much less ambiguous than interpretation based on % *H*, and we have more confidence in the former.

After completion of this work (Damha & Ogilvie, 1986), our attention was directed to a recent paper by Remaud et al. (1987) in which the temperature dependence of the chemical shifts of $A^{p}G$, A^{G}_{C}, and A^{G}_{U} is described. The conclusion that the purine bases in A^{G}_{C} and A^{G}_{U} are stacked is confirmed and extended by our CD and NMR investigations of these and other branched trinucleotides. These workers concluded that the base(2′–5′)base interaction in A^{G}_{U} and A^{G}_{C} is preferred over that of base(3′–5′)base due to an "overwhemingly more preferred" purine–purine versus purine–pyrimidine stacking. We have found, however, that the base(2′–5′)base interaction occurs in all branched trinucleotides, e.g., A^{C}_{G} and A^{G}_{C}, irrespective of the composition of the stacking bases. A CPK molecular model of A^{U}_{U} was constructed with the adenine-(3′–5′)uracil stacking. These bases can be brought together only when the C3′–O3′ and C2′–O2′ simultaneously adopt the ϵ^{t} conformation. It becomes immediately evident that, when the phosphate groups adopt this orientation, there exist short intramolecular contacts between the furanose oxygen (O1′) of the 3′-uridine residue and the charged 2′-phosphate group, an interaction that is totally absent in the adenine(2′–5′)uracil stack (Figure 9). This unfavorable situation may favor the base(2′–5′)base over base(3′–5′)base interaction, particularly in aA^{U}_{U} where the steric constraints between the 3′-residue and the 2′-phosphate group become more important.

While A^{U}_{U} and A^{G}_{G} possess most conformational features in common, there are some differences when examined in sufficient detail. Comparison of the sugar puckering of the adenosine and of the 2′-residue in A^{U}_{U} with those in A^{G}_{G} demonstrates a larger conformational purity in the former. In addition, elevation of temperature from 22 to 70 °C slightly depopulated the ^{2}E conformer of the adenosine furanose in A^{U}_{U} but had no effect on the ^{2}E–^{3}E equilibrium of that in A^{G}_{G}. It is conceivable that the S–N stack of the adenosine and 2′-uridine is the predominant stacked state of the furanoses in A^{U}_{U} at low temperature, whereas in A^{G}_{G} "mixed" stacked states (e.g., S–N and S–S with some N–S and N–N) may coexist.

We believe that branched trinucleoside diphosphates contain much of the conformational information essential for understanding the conformation of higher chain length branched RNA fragments.[9]

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[9] Preliminary results on the conformational analysis of $U3'_{p}5'A^{U}_{U}$ do indicate that most of the conformational preferences of A^{U}_{U} are preserved upon this single unit chain extension.

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