Conformational Properties of Purine–Pyrimidine and Pyrimidine–Purine Dinucleoside Monophosphates†

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ABSTRACT: The detailed conformational features and dynamics of heterodinucleoside monophosphates ApU, ApC, GpU, GpC, UpA, CpA, UpG, and CpG have been studied in aqueous solution by high field nuclear magnetic resonance (NMR) spectroscopy. Analysis of the resultant NMR parameters leads to a number of discernible trends throughout the series. Thus the ribose rings of the dimers exist as equilibrium mixtures of C(2′)-endo(^{2}E) ⇄ C(3′)-endo(^{3}E) conformers with a proclivity for the ^{3}E pucker in most cases; the C(4′)–C(5′) bonds of both nucleotidyl units show significant preference (74–96%) for a gg conformation and the dominant conformer (85–89%) about C(5′)–O(5′) is g′g′. Orientation about the C(3′)–O(3′) bond is coupled to the ribose conformational equilibrium and the system exists with a bias for the ^{3}Eg^- coupled conformation in which the H(3′)–C(3′)–O(3′)–P dihedral angle occupies the narrow range of 33–35°. Dimerization, on the average, causes about 10% increase in gg and g′g′ populations and the g^- domain becomes increasingly populated about the C(3′)–O(3′) bond. The ribose equilibrium ^{2}E ⇄ ^{3}E shifts in favor of ^{3}E upon dimerization, the effect being very conspicuous for the pu-py series (~40 → 60%) and less noticeable for the py-pu systems (~47 → 58%), clearly suggesting a correlation between sequence and ribose conformational equilibrium. The temperature and dimerization data for the heterodinucleoside monophosphates show that the transition ^{2}E → ^{3}E is directly related to χ_{CN} changes induced by dimerization and stacking. Analysis of the ribose coupling data shows that the percentage populations of stacked species vary from dimer to dimer with GpC displaying a maximum of 45% stacked population and UpG about 10%. However, in general, the pu-py dimers show a higher preference (27–45%) for stacked conformations than py-pu dimers (10–25%). It is proposed that the pronounced deshielding of H(5′) of the 5′-nucleotidyl units upon dimerization is associated with the presence of right-handed stacks (g^-g^-), whereas the chemical shift trends of H(5′) and H(5″) of 3′-nucleotidyl units are due to the presence of left-handed stacks (g^+g^+) in all the dimers. In pu-py dimers, the population of the g^-g^- species is found to be greater than that of g^+g^+. Also the population of g^-g^- stacks in pu-py dimers is generally greater than in their corresponding matched py-pu dimers. Thus the base sequence has not only an explicit effect on the overall populations of the stacked species, but also on the handedness of the stacks. The present results further confirm the interdependence of conformational bonds throughout the nucleotidyl framework.

An understanding of nucleic acids (RNA, DNA) and their different functions in various stages of gene expression requires a knowledge of their three-dimensional structures and the forces responsible for their structures; however, the complexity of large biological molecules limits the detail in which the role of the various forces can be defined. A greater insight may be gained by examining small segments and model compounds such as dinucleoside monophosphates. Along this line, a comprehensive study of the conformational properties of the dipurine and dipyrimidine dinucleoside monophosphates ApA, ApG, GpA, UpU, CpC, UpC, and CpU was recently reported from our laboratories (Lee et al., 1976), and the model that emerged from this study is that of a coupled set of conformational parameters and interdependent structural changes linked to base stacking interactions.

This work has now been extended to include the heterodinucleoside monophosphates ApU, ApC, GpU, GpC, UpA, CpA, UpG, and CpG. One of the earlier and most extensive studies of these dimers was reported by Ts'o and co-workers (1969). They derived a general conformational model in which the bases had an anti conformation with respect to the sugar rings and were stacked in a right-handed manner. There were additional attempts by Bangerter and Chan (1969) to characterize the intramolecular base-stacking interactions by investigating the concentration and temperature behaviors of ApC and CpA. Although these studies provided important information about the structure of the dimers, further progress in defining the ribose ring and phosphodiester backbone conformations and the geometry of the stack was hampered because of the difficulty in analyzing the complex NMR[1] spectral patterns which originate from ribose ring protons of the dimers. We have overcome these difficulties by a combination of the use of high-frequency Fourier transform NMR, structural simulations, and selective deuteration of residues. Hence, in this paper we are able to present a systematic and comprehensive proton NMR study and conformational deductions for

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[1] Abbreviations used: CW, continuous wave; FT, Fourier transform; TMA, tetramethylammonium chloride; UV, ultraviolet; NMR, nuclear magnetic resonance; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; for convenience in presentation of results and discussion, the following shorthand has been adopted: pu-pu, purine–purine; py-py, pyrimidine–pyrimidine; py-pu, pyrimidine–purine; and pu-py, purine–pyrimidine; pu- or py-, 3′-nucleotidyl unit; -pu or -py, 5′-nucleotidyl unit.

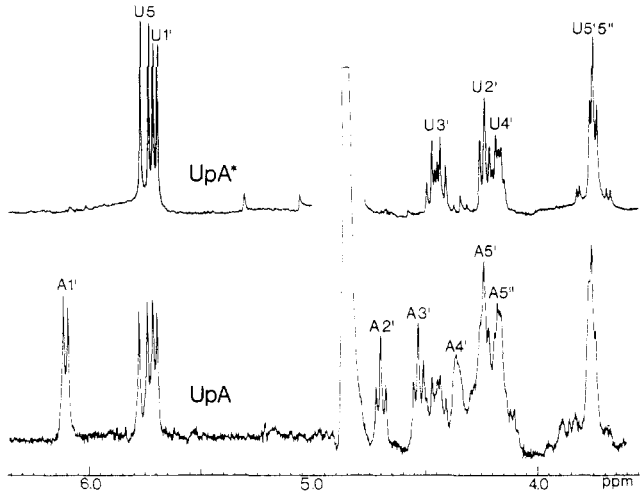


FIGURE 1: The 220-MHz ^1H NMR spectra of UpA* (top) and UpA (bottom), 20 °C, 0.01 M, pH 7.0. The base protons are not shown.

all purine–pyrimidine and pyrimidine–purine dinucleoside monophosphates.

Experimental Section

Materials. The 3′- and 5′-mononucleotides and dinucleoside monophosphates were purchased from Sigma Chemical, P-L Biochemicals, and Collaborative Research in the acid or salt form. All of the compounds were used without further purification. The nucleotides were dissolved in 100% D_2O containing a trace of sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ (TSP), or tetramethylammonium chloride (TMA) lyophilized and dissolved once more in 100% D_2O. The pD of the solution was then measured with a Beckman Research pH meter Model 1019 (pD = meter reading + 0.4) and adjusted to ~7.0 by addition of concentrated DCl or NaOD. Final concentrations ranged from 0.01 to 0.03 M for monomers and dimers and are sufficiently low to minimize intermolecular effects on shifts.

Synthesis of Deuterated Derivatives. A number of selectively deuterated heterodinucleoside monophosphates such as UpA* and *GpU (the asterisk indicates the deuterated residue) were synthesized so as to provide complete and unambiguous assignments for spectra of both classes of mixed dimers. Syntheses were achieved by both enzymatic and chemical procedures described in detail elsewhere (Kondo and Danyluk, 1972; Kondo et al., 1973). Deuterated nucleotides were isolated from RNA fractions of fully deuterated blue-green algae, *Synchococcus lividus,* and then condensed with the appropriate protonated nucleotide derivative to yield the dimers. Comparison of spectra for protio and selectively labeled compounds along with spectra of the respective monomers allowed a direct assignment of all the signals.

Measurement of Spectra. Proton spectra were recorded with a variety of spectrometers (Varian 100, 220, 300 and Bruker 270) in CW, FT, and ^1H-[^{31}P] modes (Lee et al., 1976). All of the spectra were measured at 20 ± 2 °C and several dimers and constituent monomers were also recorded at elevated temperatures. Bruker and Varian variable temperature accessories were used for temperature control. Spectra were calibrated by the audio side-band technique using a Hewlett-Packard 4204A oscillator and signal positions were measured relative to internal TSP or TMA, with an accuracy of 0.005 ppm. The chemical shifts with respect to TMA were adjusted, i.e., δTSP = δTMA + 3.206 ppm, and the δ values are reported with respect to TSP. In order to minimize the effects of differences in phosphate ionization state, spectra for 3′ and 5′ monomers were measured at pD of 5.5 ± 0.3 and dimers at pD = 7.4. At these pD values the phosphate group has a single negative charge in both monomers and dimers.

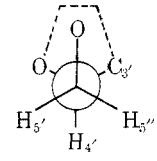
Analysis of Spectra. An initial set of NMR parameters was obtained directly from the observed spectra. Since the spectra in most cases were not first order, a further refinement was made by simulations using a Varian 620i six-spin NMR simulation program. A final iterated set of parameters was then derived using LAOCOON III or NMREN and NMRIT iterative programs.

It should be noted that both the measurement of spectra and subsequent analyses were conducted independently at two different laboratories (Argonne and SUNY, Albany). The results reported in Tables I to IV represent an average of those obtained in each laboratory. Agreement between the independent sets of parameters for all of the monomers and dimers was surprisingly good, ±0.01 ppm for shifts and ±0.2 Hz for couplings.

Results

Assignments. Figure 1 shows the 220-MHz spectra for 0.01 M UpA (NH_4^+ salt), pD = 7.4, and UpA* in D_2O at 20 °C. The -pA H(8), H(2), and Up-H(6) base proton signals are located at lower field and have not been included in the figure. Signal assignments in the upper spectrum refer to Up- protons. As is apparent, the problem of overlap arises most severely in the region of the 2′, 4′ Up- and 5′5″ -pA proton signals and is resolved by appropriate deuterated derivatives. Further confirmations of signal assignments for individual nucleotidyl units were made by consideration of splitting patterns, chemical shifts, ^1H-[^1H], ^1H-[^{31}P] decoupling experiments and spectral simulations.

Spectra for other py-pu dimers, CpG, CpA, and UpG are similar to UpA except for the near magnetic equivalence of the 4′ and 5′ signals of the -pu residue in CpG and CpA. Another feature of the UpA spectrum is a substantial shift nonequivalence of 5′,5″ protons at the 5′ phosphate terminal. This nonequivalence is characteristic of all ribonucleoside monophosphates and ranges from 0.06 to 0.30 ppm. The protons at C(5′) are designated and assigned as shown below so that H(5′) is always gauche and H(5″) trans to O(4′) (ribose ring oxygen). Under this system of nomenclature, the H(5′) signal of the 5′-nucleotidyl unit appears at a lower field relative to H(5″)



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(Remin and Shugar, 1972; Davies and Rabczenko, 1975) and the H(5″) signal of the 3′-nucleotidyl unit appears at a lower field relative to H(5′) (Son and Guschlbauer, 1975). The rationale for this is discussed in the section on O(3′)-P and O(5′)-P conformations. It is assumed that no "cross-over" of signals occurs upon dimerization so that the above labeling is also used for monomers in calculating dimerization shifts and changes in coupling constants.[2]

An example of a typical pu-py spectrum is given in Figure

[2] In a detailed temperature-dependence study on several dimers (unpublished work), no cross-over was observed of H(5′), H(5″) signals as the chemical shifts approached those for the monomers with increasing temperature.

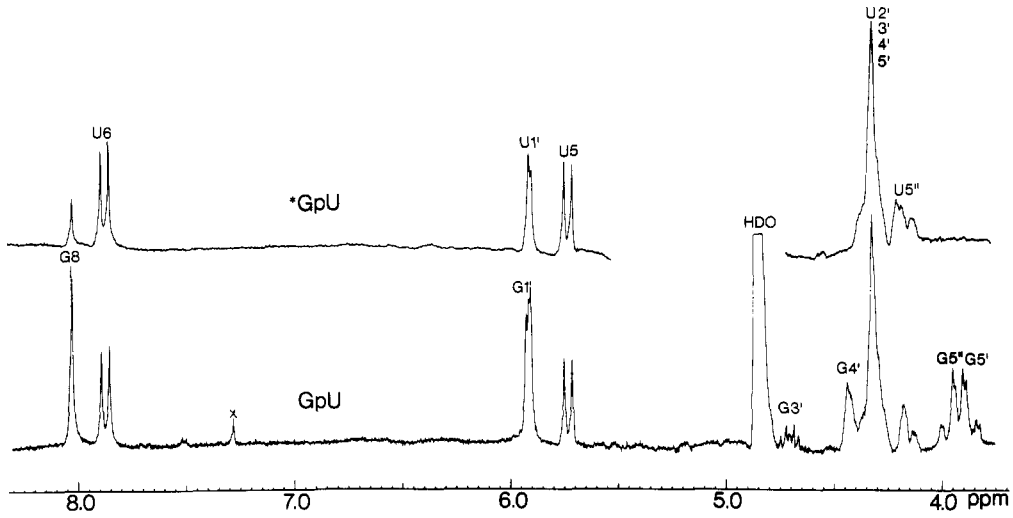

FIGURE 2: The 220-MHz ^{1}H NMR spectra of *GpU (top) and GpU (bottom), 20 °C, 0.01 M, pH 7.0.

2. The lower spectrum is for a 0.01 M solution of GpU while the upper spectrum shows its deuterated analogue, *GpU. Assignment of signals to individual nucleotides follows directly from comparison of the two spectra. Since H(8) of the guanine base is exchangeable, the observed signal appears with diminished intensity in the *GpU spectrum. The Gp- H(2′) signal is not identified in Figure 2 since it is concealed under the HDO signal, but is clearly observable at higher temperatures. Other features to note are the near chemical shift equivalence of -pU 2′, 3′, 4′, and 5′ protons and a substantial 0.12-ppm deshielding of H(5″) relative to H(5′) at the 3′ free terminal (Gp-). The latter is characteristic of pu-py and py-py (Lee et al., 1976) dimers, but not of the other dimer sequences. Small differences are noted in the spectra for the other pu-py molecules compared with that of GpU. For example, the H(2′) signal is well separated and shifted upfield by ~0.08 ppm from nearly overlapped H(3′) and H(4′) signals for -pC of both GpC and ApC. Also the pu- H(1′) signal is generally found at lower field compared with py with the exception of GpC where the order is reversed. This assignment was established with the aid of *GpC.

The 300-MHz observed and computer-simulated spectra of UpA and ApU at probe temperature are shown in Figure 3 and that of CpG at 80 °C in Figure 4 and are typical of the excellent agreement between observed and calculated spectra for all dimers.

Chemical Shifts and Coupling Constants. Chemical shifts of the base and ribose ring proton signals for the dimers at 0.01–0.03 M, pD 7.4, and 20 °C are listed in Table I. The shifts for base and anomeric H(1′) signals are generally in agreement with values reported earlier by Ts'o and co-workers (Ts'o et al., 1969). In the present studies, however, shifts are reported for *all* protons of the dimers and these reveal several interesting sequence-related effects not available from base data alone. For example, for matched pairs of pu-py and py-pu, the H(2′) and H(3′) signals of the 3′-nucleotidyl fragment (pu-, py-) are always found downfield from those in -pu or -py, while H(1′), H(5′), H(5″) signals of pu- and py- are located upfield from those for -pu and -py, Table I. Also, H(4′) shifts of purines are at lower field in the 3′-nucleotidyl unit (pu- > -pu), while for pyrimidines the situation is reversed, i.e., $\delta_{4'}$ of -py is at lower field than that of py-. These trends hold for all pairs of ApU-UpA, ApC-CpA, etc. dimers and differ from shift trends in corresponding monomers and homodimers.

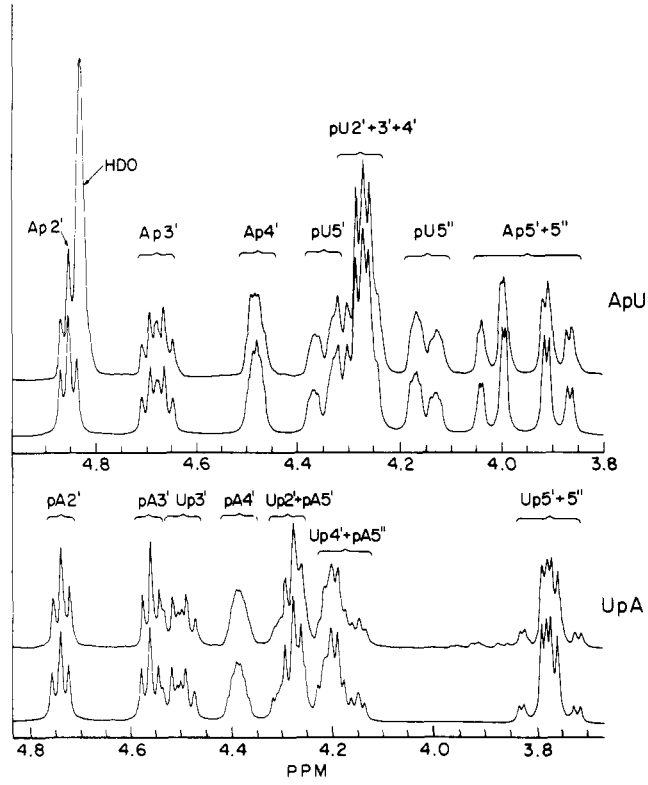

FIGURE 3: The 300-MHz ^{1}H NMR spectra of ApU (top) and UpA (bottom) and the corresponding simulations, 20 °C, 0.02 M, pH 7.0. The base and 1′ proton signals are not shown.

Trends for purine base protons follow the pattern found earlier for mononucleotides and purine dinucleoside monophosphates (Ts'o et al., 1969; Hruska and Danyluk, 1968; Davies and Danyluk, 1974, 1975; Lee et al., 1976); i.e., H(2) and H(8) are at higher field in pu- than in -pu. Thus $\delta_{H(2)}$ = 8.181 ppm in ApU and 8.225 ppm in UpA. However, $|\delta_{2(8)}(\text{-pu}) - \delta_{2(8)}(\text{pu-})|$ is, on the average, smaller in the heterodimers compared with the homoseries. For py base protons, the H(5) and H(6) of py- are downfield from -py in all matched pairs of heterodimers with the exception of H(6) in GpU/UpG and GpC/CpG pairs. A purine nucleotidyl unit in a 3′ position thus has a greater shielding effect on H(5) of -py than a 5′-nucleotidyl unit on H(5) of py-.

TABLE I: Chemical Shifts[a] of Dinucleoside Monophosphates in D_2O.[b]

Nucleotide	Temp (°C)	1′	2′	3′	4′	5′	5″	Δ5′5″[c]	2(5)	8(6)
ApU Ap-	20	6.054	4.838	4.648	4.468	3.895	4.014	0.119	8.181	8.311
-pU		5.745	4.239	4.292	4.249	4.339	4.136	0.203	5.586	7.754
ApC Ap-	20	6.031	4.833	4.643	4.460	3.910	4.041	0.131	8.086	8.337
-pC		5.722	4.146	4.244	4.238	4.381	4.133	0.248	5.675	7.716
ApC Ap-	80	6.167	4.880	4.788	4.479	3.889	3.977	0.088		
-pC		5.909	4.269	4.304	4.284	4.319	4.193	0.256		
GpU Gp-	20	5.906	4.832	4.696	4.422	3.870	3.968	0.098		8.002
-pU		5.900	4.280	4.327	4.309	4.333	4.152	0.181	5.716	7.860
GpC Gp-	20	5.855	4.820	4.617	4.387	3.886	4.019	0.133		8.027
-pC		5.877	4.193	4.280	4.267	4.432	4.137	0.295	5.648	7.793
UpA Up-	20	5.725	4.255	4.472	4.189	3.748	3.798	0.050	5.762	7.756
-pA		6.100	4.710	4.540	4.375	4.264	4.155	0.109	8.225	8.411
CpA Cp-	20	5.699	4.307	4.428	4.216	3.798	3.889	0.091	5.804	7.723
-pA		6.098	4.595	4.523	4.358	4.313	4.161	0.152	8.235	8.447
UpG Up-	20	5.789	4.300	4.470	4.190	3.733	3.733	0.000	5.793	7.790
-pG		5.907	4.742	4.534	4.318	4.233	4.165	0.068		8.043
CpG Cp-	20	5.735	4.318	4.437	4.213	3.770	3.820	0.050	5.869	7.759
-pG		5.901	4.638	4.516	4.316	4.293	4.164	0.129		8.023
CpG Cp-	80	6.017	4.341	4.533	4.242	3.787	3.853	0.066		
-pG		6.070	4.740	4.516	4.339	4.244	4.188	0.056		

[a] Shifts are given in ppm (δ) relative to internal TSP and are accurate to ±0.005 ppm. [b] pD = 7.4; concentration, 0.01–0.03 M. [c] $\Delta 5'5'' = \delta_{5'} - \delta_{5''}$.

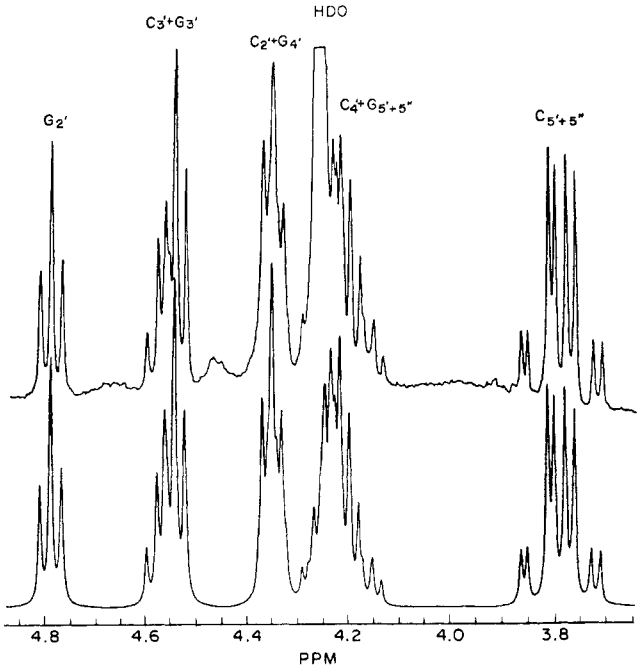

FIGURE 4: The 270-MHz ^1H NMR spectrum of CpG at 80 °C, 0.02 M, pH 7.0, and the corresponding simulation.

As with the homopurines, the 5′,5″ protons of -pu and -py show a rather striking behavior (Lee et al., 1976). In all instances these shifts are not only to low field from H(5′),H(5″) of pu-, py- (loc. cit.), but their nonequivalence, $\delta_{5'} - \delta_{5''}$, is much greater in the 5′-nucleotidyl position, i.e., 0.07–0.30 ppm vs. 0.0–0.13 ppm. The increased nonequivalence arises to a large extent because of a preferential deshielding of H(5′) (-pu, -py) (see following section and Lee et al., 1976).

Within the individual series, the largest variability in shifts (~0.20 ppm) is observed for H(1′) in both pu-py and py-pu, irrespective of 3′ or 5′ fragment. A sizeable shift range (0.13–0.15 ppm) is also seen for H(2′), but only in 5′-nucleotidyl fragments. The remaining protons show comparatively smaller variations within each series. A number of interesting features also stand out in a comparison of shift data for homo- and heterodimers. For the series, pu-pu, pu-py, py-pu, py-py, the pu base protons are displaced to lower field in order pu-pu → pu-py → py-pu, while those of py vary as pu-py → py-pu → py-py in all cases with the exception of H(6) in certain dimers. A similar trend is followed by 1′ protons but not by the other ribose protons.

Spectra for several of the dimers were also obtained at high temperatures in order to determine any thermally induced conformational changes. Included in Table I are the chemical shifts of two dimers, ApC and CpG, at 80 °C. As can be seen, most of the base and ribose signals shift downfield with increasing temperature, the exception being H(5′) of -pC, and -pG and both H(5′) and H(5″) of Ap- which show upfield shifts.[2] The largest changes occur for base and ribose 1′, 2′, and 3′ protons in both classes of dimers while the smallest effect is seen for H(4′).

For ribose rings, the coupling constant $J_{1'2'}$ is less than $J_{3'4'}$ in all of the dimers studied with variations in these couplings amounting to as much as 3 Hz across the series. On the other hand, $J_{2'3'}$ and $J_{1'2'} + J_{3'4'}$ are essentially constant with average values of 5.3 and 9.5 Hz, respectively, being identical with those for homodimers (Lee et al., 1976). A close agreement is noted between coupling constants of ApU and the values obtained recently by Altona (Altona et al., 1974; Altona, 1975) for N^6-methyl derivatives of ApU. A definite sequence dependence is seen for $J_{1'2'}$ ($J_{3'4'}$) with $J_{1'2'}$(py) < $J_{1'2'}$(pu) for a given dimer and $J_{1'2'}$(pu-py) < $J_{1'2'}$(py-pu) for both nu-

TABLE II: Coupling Constants[a] for Dinucleoside Monophosphates in D_2O.[b]

Nucleotide	Temp (°C)	1′2′	2′3′	3′4′	4′5′	4′5″	3′P	4′P	5′P	5″P	5′5″	5 6
ApU Ap-	20	3.8	5.2	5.4	3.0	2.1	8.4				−13.1	
-pU		3.5	5.2	5.6	2.2	2.3		2.0	3.5	3.7	−11.9	8.2
ApC Ap-	20	3.6	5.3	6.1	3.3	2.2	8.7				−12.9	
-pC		2.5	5.2	7.1	2.1	2.6		2.5	3.6	3.6	−11.4	7.7
ApC Ap-	80	5.4	5.3	4.2	4.2	2.8	8.0				−13.0	
-pC		3.4	5.2	5.8	2.8	4.2		1.8	5.2	5.2	−12.0	
GpU Gp-	20	4.4	5.3	4.6	3.5	2.4	8.4				−12.9	
-pU		3.5	5.2	5.9	2.2	2.6		2.0	4.0	4.0	−11.2	8.2
GpC Gp-	20	2.6	5.3	6.8	4/		2.3	8.6				−13.0
-pC		2.2	5.2	7.5	2.2	2.2		2.0	3.2	3.3	−11.5	7.7
UpA Up-	20	4.3	5.2	5.0	3.9	2.6	8.1				−12.9	8.2
-pA		4.6	5.2	4.9	2.6	3.4		2.0	4.0	4.3	−11.8	
CpA Cp-	20	3.1	5.3	6.8	3.7	2.3	8.4				−12.9	7.9
-pA		4.2	5.1	5.4	2.0	3.0		2.0	3.8	3.8	−11.8	
UpG Up-	20	4.8	5.2	5.1	3.2	3.2	8.2				−12.9	8.1
pG		5.0	5.2	4.8	2.6	3.3		2.0	4.0	4.2	−11.5	
CpG Cp-	20	3.2	5.1	6.7	3.2	2.8	8.3				−13.0	7.9
-pG		4.3	5.0	5.4	2.4	3.0		2.0	3.3	3.9	−11.2	
CpG Cp-	80	4.6	5.3	5.0	4.6	3.0	8.3				−12.8	
-pG		5.2	5.3	4.6	2.8	4.4		2.0	4.8	5.2	−11.4	

[a] Coupling constants are accurate to ±0.1–0.2 Hz. [b] Solution conditions are the same as in Table I.

cleotidyl fragments, Table II. The first relationship is particularly useful for assignment of H(1′) signals for heterodimers since $J_{1'2'}$(py) is always less than $J_{1'2'}$(pu) irrespective of base sequence.

A number of interesting trends are also revealed for couplings along the ribose phosphate backbone. Two sets of couplings across C(4′)–C(5′) are present in dimers, one directly involved in the phosphodiester linkage and the other present in the exocyclic carbinol group. For the series as a whole, $J_{4'5'}$ of the first set varies over a narrower range (2.1–2.6 Hz) than $J_{4'5''}$ (2.3–4.0 Hz), but in every hetero- and homodimer analyzed thus far, the coupling of H(4′) to the downfield signal (H(5′)) is *always smaller* regardless of nucleotidyl type, -pu or -py. The remaining backbone couplings, $J_{5'P}$ and $J_{5''P}$, range from 3.5 to 4.0 Hz and 3.6 to 4.3 Hz, respectively, with no sequence effect of note. Similarly, no sequence trend is discernible for either $J_{3'P}$ or $J_{4'P}$ both of which are nearly constant in the dimer series.

An elevation in temperature from 20 to 80 °C produces substantial effects on a number of conformationally important couplings. ApC and CpG show an increase of $J_{1'2'}$ that is matched by a decrease in $J_{3'4'}$; $J_{2'3'}$ and $J_{1'2'} + J_{3'4'}$, on the other hand, are unchanged. Increases of up to 50% also occur for couplings along the ribose phosphate backbone, $J_{4'5'}$, $J_{4'5''}$, $J_{5'P}$, and $J_{5''P}$.

Dimerization Effects. Dimerization effects on chemical shifts and coupling constants (monomer–dimer) were calculated for all of the heterodimers and the results compiled in Tables III and IV. Although no NMR parameters for mononucleotides at pD 5.4 are given in Tables III and IV, the values may be inferred from Tables I–IV. Positive $\Delta\delta$ values in Table III denote an upfield chemical shift, while positive ΔJ values in Table IV represent a decrease in spin–spin coupling constants upon dimerization.

The dimerization shifts of virtually all the base and ribose ring protons are positive, except for the exocyclic 5′ and 5″ protons which exhibit both upfield and downfield shifts. Significant trends are obtained in each class of dimers. For pu-py dimers, dimerization produces a much greater effect on -py base protons than on pu, i.e., $\Delta\delta_{\text{-py}}$ (0.13–0.48 ppm) $\gg$ $\Delta\delta_{\text{pu-}}$ (0.02–0.09 ppm) with upfield shifts being greater for H5, $\Delta\delta_{H(5)}$ (0.23–0.48 ppm) > $\Delta\delta_{H(6)}$ (0.13–0.33 ppm). In ribose rings, H(1′) shows the most pronounced upfield shifts with changes in -py moieties again being greater (0.09–0.28 ppm) than in pu- (0.04–0.09 ppm). The 4′ protons are affected least by dimerization (0.005 to 0.05 ppm) and the dimerization shifts of H(2′) and H(3′) are intermediate (0.01–0.18 ppm) between those of H(1′) and H(4′). The trend $\Delta\delta_{\text{-py}} > \Delta\delta_{\text{pu-}}$ is maintained for 2′ and 4′ protons, but H(3′) shows a reversal so that $\Delta\delta_{\text{pu-}}$ (0.08–0.15 ppm) > $\Delta\delta_{\text{-py}}$ (0.03–0.10 ppm). A surprisingly large deshielding, ranging between 0.17 and 0.24 ppm, occurs for 5′, and 0.04–0.06 ppm for 5″ protons of the ribose phosphate backbone, the former showing a significantly greater change and producing an increased magnetic nonequivalence of the two protons. For 5′ and 5″ protons of the 3′-nucleotidyl fragment (pu-), the dimerization shifts are less marked, but once more both protons are deshielded with $|\Delta\delta_{H(5'')}| > |\Delta\delta_{H(5')}|$.

Base protons of py-pu dimers are shifted upfield in the same manner as those in pu-py, i.e., $\Delta\delta_{\text{py-}}$ (0.11–0.28 ppm) > $\Delta\delta_{\text{-pu}}$ (0.02–0.09 ppm) and $\Delta\delta_{H(5)}$ (0.12–0.28 ppm) > $\Delta\delta_{H(6)}$ (0.11–0.18 ppm), but the magnitudes of the changes are noticeably smaller. For ribose protons, dimerization effects on H(1′) ($\Delta\delta_{\text{py}}$ ~0.17–0.25 ppm and $\Delta\delta_{\text{-pu}}$ ~ 0.04 ppm) are greater than for remaining protons, with H(3′) and H(4′) being least affected ($\Delta\delta$ ~ 0.02–0.12 ppm). The largest shift perturbations again occur for pyrimidine nucleotides, $\Delta\delta_{\text{py}}$ (1′, 2′, 3′, and 4′) > $\Delta\delta_{\text{pu}}$ (1′, 2′, 3′, and 4′). The backbone protons

TABLE III: Dimerization Shifts, δ(monomer) − δ(dimer), ppm.

Nucleotide	1′	2′	3′	4′	5′	5″	Δ[a]	2(5)	8(6)
ApU Ap-	0.050	0.036	0.144	0.031	0.013	−0.056	0.069	−0.005	−0.021
-pU	0.245	0.124	0.100	0.032	−0.179	−0.046	0.133	0.352	0.237
ApC Ap-	0.064	0.041	0.149	0.039	−0.002	−0.083	0.081	0.090	−0.005
-pC	0.277	0.175	0.093	0.045	−0.183	−0.040	0.143	0.449	0.326
GpU Gp-	0.043	0.004	0.075	−0.004	−0.005	−0.053	0.048		0.035
-pU	0.090	0.083	0.031	−0.028	−0.173	−0.062	0.111	0.234	0.131
GpC Gp-	0.094	0.016	0.154	0.031	−0.021	−0.104	0.083		0.010
-pC	0.122	0.128	0.057	0.016	−0.234	−0.044	0.190	0.476	0.249
UpA Up-	0.235	0.204	0.100	0.111	0.100	0.130	−0.030	0.153	0.149
-pA	0.037	0.045	−0.029	0.018	−0.129	−0.020	0.109	0.029	0.084
CpA Cp-	0.253	0.126	0.124	0.077	0.062	0.059	0.003	0.280	0.183
-pA	0.039	0.160	−0.012	0.035	−0.178	−0.026	0.152	0.019	0.048
UpG Up-	0.171	0.159	0.102	0.110	0.115	0.195	−0.080	0.122	0.115
-pG	0.037	0.013	−0.039	0.032	−0.109	−0.041	0.068		0.079
CpG Cp-	0.217	0.115	0.115	0.080	0.090	0.128	−0.038	0.215	0.147
-pG	0.043	0.117	−0.021	0.034	−0.169	−0.040	0.129		0.089

[a] $\Delta = [(\delta_{5'} - \delta_{5''})_{\text{dimer}} - (\delta_{5'} - \delta_{5''})_{\text{monomer}}]$.

TABLE IV: Dimerization Effects on Coupling Constants [J(monomer) − J(dimer), Hz].

Nucleotide	1′2′	2′3′	3′4′	4′5′	4′5″	3′P	4′P	5′P	5″P	5′5″	5 6
ApU Ap-	2.4	0.0	−2.5	0.3	0.3	−0.5				0.4	
-pU	1.4	−0.1	−1.3	0.1	0.5		0.1	0.8	1.3	0.1	0.0
ApC Ap-	2.6	−0.1	−3.2	0.0	0.2	−0.8				0.2	
-pC	1.6	−0.2	−2.0	0.3	1.2		−0.5	0.6	1.5	−0.4	−0.1
GpU Gp-	1.4	−0.1	−1.5	0.1	0.7	−0.7				0.2	
-pU	1.4	−0.1	−1.6	0.1	0.2		0.1	0.3	1.0	−0.6	0.0
GpC Gp-	3.2	−0.1	−3.7	−0.4	0.8	−0.9				0.3	
-pC	1.9	−0.2	−2.4	0.2	1.6		0.0	1.0	1.8	−0.3	−0.1
UpA Up-	0.6	0.0	0.2	0.3	0.3	0.1				0.1	0.0
-pA	1.1	0.0	−1.2	0.5	0.3		−0.3	0.1	0.7	−0.2	
CpA Cp-	1.1	0.0	−1.2	0.5	0.4	0.1				0.0	−0.2
-pA	1.5	0.1	−1.7	1.1	0.1		−0.3	1.2	1.2	−0.2	
UpG Up-	0.1	0.0	0.1	1.0	−0.3	0.0				0.1	0.1
-pG	1.1	0.0	−1.4	0.8	0.1		0.0	1.1	0.9	−0.3	
CpG Cp-	1.0	0.2	−1.1	0.9	−0.1	0.2				0.1	−0.2
-pG	1.8	0.2	−2.0	1.0	0.4		0.0	1.8	1.2	−0.6	

of -pu, H(5′)(5″), shift markedly downfield with an accompanying increase in magnetic nonequivalence $\Delta\delta_{5'} > \Delta\delta_{5''}$. But the situation is reversed at the 5′ free terminal (py-). Not only are both signals shifted upfield, but $\Delta\delta_{5''} > \Delta\delta_{5'}$ leading to a decrease in their shift nonequivalence.

Comparison of the two dimer classes, pu-py and py-pu, shows obvious differences in dimerization effects on shifts: (1) for the base protons, $\Delta\delta_{(\text{pu-py})} > \Delta\delta_{(\text{py-pu})}$ particularly for py protons; (2) for the H(3′) proton, $\Delta\delta_{\text{pu-}}$ (0.08–0.15 ppm) > $\Delta\delta_{\text{-py}}$ (0.03–0.10 ppm) in pu-py dimers and $\Delta\delta_{\text{py-}}$ (0.10–0.12 ppm) > $\Delta\delta_{\text{-pu}}$ (−0.03–0.01 ppm) in py-pu dimers; (3) upfield shifts of both 4′ protons are small (−0.02–0.05 ppm) in pu-py but $\Delta\delta_{4'\text{py-}}$ (0.08–0.11 ppm) is significantly greater than $\Delta\delta_{4'\text{-pu}}$ (0.02–0.04 ppm) in py-pu (this trend is also observed in pu-pu dimers); (4) although backbone H(5′) and H(5″) are deshielded in both sequences of dimers, the pu- 5′,5″ protons are *deshielded* and the magnetic nonequivalence *increased* relative to the monomers in pu-py, whereas the py- 5′,5″ protons in py-pu are *shielded* and their magnetic nonequivalence *decreased* as well.

A dominant feature of dimerization effects on couplings is a 1.0–2.4-Hz decrease in $J_{1'2'}$ and a comparable increase in $J_{3'4'}$ irrespective of dimer type, Table IV. $J_{2'3'}$ remains essentially constant. Although ΔJ values are greater (1.1–3.2 Hz) for the pu moiety than for py (0.1–1.6 Hz), the magnitudes of $J_{1'2'}$ are also smaller in the latter (see Table II). This reflects the fact that $J_{1'2'}$ of pyrimidine mononucleotides (4.1–4.9 Hz) are initially smaller than those of purine mononucleotides (5.7–6.2 Hz).

The dimerization effects on exocyclic group couplings are generally positive for 4′5′, 4′5″, 5′P, and 5″P, (0.1–1.8 Hz), and negative for 3′P of pu-py dimers. Further comparison of

TABLE V: Population Distribution of Conformers in Dinucleoside Monophosphates and Their Components.

Nucleotide	Temp (°C)	% Stacked[e]	Dimer: Ribose Ring[a] % ^3E	Dimer: Ribose Ring[a] K_{eq}[d]	Dimer: Backbone[c] % gg	Dimer: Backbone[c] % g'g'	Dimer: Backbone[c] θPH	Monomer[b]: Ribose Ring[a] % ^3E	Monomer[b]: Ribose Ring[a] K_{eq}[d]	Monomer[b]: Backbone[c] % gg	Monomer[b]: Backbone[c] % g'g'	Monomer[b]: Backbone[c] θPH
ApU Ap-	20	34 ± 3	57	1.32	89		±34	31	0.45	82		±36
-pU			59	1.44	95	85		46	0.85	89	75	
ApC Ap-	20	38 ± 2	64	1.78	85		±33	31	0.45	82		±36
-pC			75	3.00	93	85		55	1.22	77	75	
ApC Ap-	80		44	0.79	69		±36					
-pC			61	1.56	69	70						
GpU Gp-	20	27 ± 5	49	0.92	80		±34	33	0.49	72		±37
-pU			62	1.63	92	82		46	0.85	89	75	
GpC Gp-	20	45 ± 4	71	2.45	76		±33	33	0.49	72		±37
-pC			79	3.76	96	89		55	1.22	77	75	
UpA Up-	20	15 ± 3	53	1.13	74		±35	56	1.27	68		±35
-pA			51	1.04	79	80		40	0.67	77	72	
CpA Cp-	20	24 ± 2	71	2.45	79		±35	60	1.50	71		±35
-pA			57	1.33	90	84		40	0.67	77	72	
UpG Up-	20	10 ± 5	54	1.17	75		±35	56	1.27	68		±35
-pG			50	1.00	80	81		37	0.59	71	71	
CpG Cp-	20	25 ± 3	71	2.45	79		±35	60	1.50	71		±35
-pG			57	1.33	85	85		37	0.59	81	71	
CpG Cp-	80		53	1.13	63		±35					
-pG			48	0.92	67	72						

[a] Computed by using $J_{1'2'} + J_{3'4'} = 9.5$ Hz for the dimers and $J_{1'2'} + J_{3'4'} = 9.3$ for monomers. [b] Monomer data for solutions at pD = 5.4. [c] Rotamer equations used, gg = $(13.7 - \Sigma)/9.7$; g'g' = $(25 - \Sigma')/20.8$. [d] K_{eq}, $^2E \rightleftarrows {}^3E$; estimated errors in K_{eq} are ±0.2. [e] % stacked = $(J_{1'2'}(\text{monomer}) - J_{1'2'}(\text{dimer}))/J_{1'2'}(\text{monomer})$ or $(J_{3'4'}(\text{dimer}) - J_{3'4'}(\text{monomer}))/(9.5 - J_{3'4'}(\text{monomer}))$. The values are the average % stacked for the purine and pyrimidine fragments.

the two classes of dimers shows that (1) $\Delta J_{1'2'}$ values for pu-py are greater than $\Delta J_{1'2'}$ for py-pu and (2) $\Delta J_{3'\text{-P}}$ values for pu-py dimers (−0.5 to −0.9 Hz) differ in magnitude and sign from $\Delta J_{3'\text{P}}$ values for py-pu (0 to 0.2 Hz).

Discussion

The procedures for deriving conformational data for nucleotidyl units have been described in earlier papers of this series (Lee et al., 1976) and have been followed in the present heterodimer study.

Conformation of Ribofuronose Rings. As in the homodimer case, the furonose ring couplings can be analyzed in terms of two puckered modes ^3E and ^2E undergoing interconversion via pseudorotation, i.e., $^3E \rightleftarrows {}^2E$. Equilibrium populations of each conformer were evaluated and the % ^3E population, compiled in Table V, shows a range from a low of 50% for -pG in UpG to a high of 79% in -pC of GpC. A slightly higher ^3E population is noted for pyrimidine fragments(53–79%) as compared with purines (50–71%). In common with homodimers, a very noticeable increase in ^3E population occurs for both pu-py and py-pu on dimerization relative to corresponding mononucleotides (Table V). Similarly, a greater change appears to occur for pu nucleotides, irrespective of sequence location, than for py. There is a clear distinction in the effects of dimerization on the two classes of mixed dimers. Within the pu-py series, dimerization produces a shift to ^3E pucker in the order GpC > ApC > ApU > GpU, with pu- being more affected than -py. GpC in fact shows the highest composite ^3E population of all the mixed and homodimers, a phenomenon related to the high degree of base stacking in this dimer. It should be noted that

TABLE VI: Stacking Parameters in Mixed Dimers.

Dinucleotide	$\Delta\delta_{H(5)}$[a]	$\Delta J_{1'2'}$[b] Pyrimidine	$\Delta J_{1'2'}$[b] Purine	% Stack[d]	Δ[c]
GpC	0.476	1.9	3.2	45 ± 4	0.190
ApC	0.449	1.6	2.6	38 ± 2	0.143
ApU	0.350	1.4	2.4	34 ± 3	0.133
GpU	0.234	1.4	1.4	27 ± 5	0.111
CpA	0.280	1.1	1.5	24 ± 2	0.152
CpG	0.215	1.0	1.8	25 ± 3	0.129
UpA	0.153	0.6	1.1	15 ± 3	0.109
UpG	0.122	0.1	1.1	10 ± 7	0.068

[a] $\delta_{H(5)}(\text{monomer}) - \delta_{H(5)}(\text{dimer})$, ppm. [b] $J_{1'2'}(\text{monomer}) - J_{1'2'}(\text{dimer})$, Hz. [c] $[(\delta_{5'} - \delta_{5''})_{\text{dimer}} - (\delta_{5'} - \delta_{5''})_{\text{monomer}}]$ for the 5'-nucleotidyl unit. [d] From Table V.

the shift to ^3E in pu- fragments takes place from corresponding monomers which are predominantly ^2E, whereas for -py fragments the starting monomers have a significantly higher ^3E population. In contrast, for the py-pu series, the effects of dimerization on $^2E \rightleftarrows {}^3E$ are not nearly as noticeable with the largest shift to ^3E occurring for CpG and CpA and smaller changes for the others. A further interesting feature relates to changes in matched pairs, i.e., pu-py and py-pu; in *all cases* the pu-py member shows a greater $^2E \rightarrow {}^3E$ shift than py-pu. Another perspective on dimerization effects is given by a comparison of $\Delta J_{1'2'}$ $(J_{1'2'}(\text{monomer}) - J_{1'2'}(\text{dimer}))$, Table VI. Again two ranges are evident with pu-py showing greater

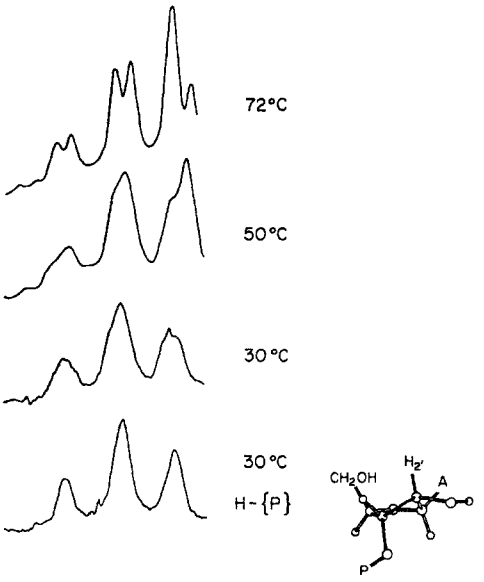

FIGURE 5: Temperature dependence of the H-2′ signal of Ap- in ApU. The bottom figure is the phosphorus decoupled spectrum.

$\Delta J_{1'2'}$ magnitudes for both pu- and py- than is true for py-pu. The $\Delta J_{1'2'}$ values for homodipurines (mean of 3′ and 5′ fragments) range from 1.7 to 2.2 Hz, while those for homodipyrimidines range from 0.7 to 1.3 Hz (Lee et al., 1976). These values lie in the mid-region observed for the heterodimers. Thus, there is a clear correlation between sequence and extent of ^{3}E pucker across the entire series of dinucleoside monophosphates. In contrast, the puckering amplitudes exhibit no base sequence dependence and remain relatively constant throughout the series of homo- and heterodimers, i.e., $J_{2'3'} = 5.2 \pm 0.2$ Hz and $J_{1'2'} + J_{3'4'} = 9.5 \pm 0.5$ Hz. Furthermore, no significant changes occur in the puckering amplitudes upon dimerization.

An elevation in temperature produces a significant shift in ribose conformer equilibrium to the ^{2}E form in both pu-py and py-pu, similar to the effects seen for homodimers. The change is more pronounced in 3′-nucleotidyl fragments irrespective of base and is apparently general throughout the entire dimer series. Dimerization thus appears to always produce a greater conformational rearrangement of the 3′-nucleotidyl unit, at least within temperature ranges encompassed in the present studies.

Backbone Conformation.[3] (a) C(4′)-C(5′) (ψ_1, ψ_2) and C(5′)-O(5′) (ϕ_2). The population distribution of conformers about C(4′)-C(5′) and C(5′)-O(5′) bonds was calculated using expressions developed elsewhere (Lee and Sarma, 1977), and a listing of gg (ψ_1, ψ_2, = 60°) and g′g′ (ϕ_2 = 180°) for monomeric components and dimers is compiled in Table V. The data reveal that, in the 3′ and 5′ mononucleotides as well as in all the dimers, the C(4′)-C(5′) bonds show a distinct preference for the gg conformation. Comparison of matched pairs pu-py and py-pu demonstrates that for a given base the -py shows a greater preference for the gg orientation than py-. This is also true of dipyrimidines and the component mononucleotides. In contrast the gg populations in -pu and pu- fragments of matched heterodimers are comparable, as they are in the component mononucleotides. In dipurines, the 3′ residue shows a greater degree of preference for the gg orientation than the 5′ part. No general correlation of the type postulated by Hruska et al. (1974) exists between the gg population and the ribose ring conformation. Dimerization, on the average, causes an increase of ~10% in gg population in the collection of molecules examined and in this respect the heterodimers differ noticeably from the homodimer series (Lee et al., 1976).

The population distribution of conformers about C(5′)-O(5′) (Table V) shows a high preference for the g′g′ (ϕ_2 = 180°) orientation in both monomers and dimers irrespective of the nature of the base. The data indicate that the C(4′)-C(5′), C(5′)-O(5′) bonds in the dimers form a highly stable conformational unit throughout the entire series. However, elevation of temperature and consequent destacking produces a significant perturbation of the conformational distribution (Table V) about these bonds.

(b) C(3′)-O(3′) (ϕ_1'). The problem of identifying the favored rotamer(s) about C(3′)-O(3′), i.e., $\phi_1' = 180°$ (g^-), $\phi_1' = 300°$ (g^+), and $\phi_1' = 60°$ (t), in solution is complex since only one coupling constant, $J_{3'P}$, is available as compared with C(4′)-C(5′) and C(5′)-O(5′) bonds. Nevertheless, a narrowing of the possibilities can be arrived at as follows. Any meaningful contribution from a t conformation can be ruled out on the grounds that it involves an extremely sterically restricted juxtaposition of the two nucleotides, and on the basis of results from x-ray diffraction and theoretical calculations (Pullman et al., 1972). The three remaining possibilities are g^+, g^-, and a $g^+ \rightleftarrows g^-$ equilibrium mixture. For pure g^+ and g^- conformers the vicinal correlation $^3J_{HP} = 18.1 \cos^2\theta - 4.8 \cos\theta$ (Lee and Sarma, 1976)[4] predicts a coupling, 2.1 Hz, for both that is markedly different from the observed couplings, 8.0-8.7. Alternatively, the observed couplings can be used to calculate POCH vicinal angle ranges of ±32-36° (Table V) ($\phi_1' \sim 210$ or 270°) and ±122-125°. The latter corresponds to sterically precluded conformations, while the former lies approximately midway between torsion angles for pure g^-, g^+ and eclipsed PO(3′)-C(3′)H(3′) bonds, i.e., within g^- and g^+ domains.[5] Among the various conformational requirements for base stacking to occur is the need for C(3′)-O(3′) torsion angles to be in the range ~180-230°; ϕ_1' values >240° produce destacking. To the extent that base stacking is a significant dimer feature (vide infra), one expects meaningful populations of g^- conformers. In the extended conformations of the dimer, both g^- and g^+ rotamers should be accessible.

Previous arguments for the accessibility of g^+ for the C(3′)-O(3′) bond in mono- and dinucleoside monophosphates were based on the observation of a four bond H(2′)-P coupling. In the favorable case in which the ribose is ^{2}E and the 3′-phosphate group is g^+, an almost in-plane "W" relation exists between the phosphorus atom and H(2′) (Figure 5). Such a "W" relation would be expected to produce a long-range four-bond ^{31}P-^{1}H(2′) coupling of magnitude 2.7 Hz (Sarma et al., 1973). Four-bond couplings between H(2′) and P have been observed in the dimers at high temperatures. Figure 5 illustrates the H(2′) resonance of Ap- of ApU as a function of temperature. At 72 °C, the four-bond coupling is clearly discernible (1.1-1.2 Hz) and its residual presence (~0.4 Hz) at 30 °C is demonstrated by ^{1}H-^{31}P decoupling. The interrelation among stacking, ribose conformation, and the orientation of the C(3′)-O(3′) bond is thus clearly illustrated by the tem-

[3] The nomenclature and numbering of atoms are as described by Lee et al. (1976) and are consistent with IUPAC and IUB recommendations.

[4] The vicinal angle θ is taken to be 0° for P-O(3′) cis to C(3′)-H(3′). This corresponds to $\phi_1' = 240°$.

[5] No change in coupling would be expected for $g^+ \rightarrow g^-$ since J values in the two domains are the same, assuming that the torsion angles at $\phi_1' \sim 180°$ and 300° correspond to rotamer energy minima.

perature data on ApU. As the temperature increases from 30 to 72 °C, the population of stacked species decreases and the percentage of ^2E conformers increases for the Ap- residues; a four-bond "W" coupling between H(2′) and P then becomes clearly discernible.

In the discussion of the orientation of the C(3′)–O(3′) bond above, the model postulates an equilibrium between conformers in the g^+ and g^- domains which is coupled to the conformation of the ribofuranose ring, i.e., $^3E\ g^+ \leftrightarrows {}^3E\ g^- \rightleftharpoons {}^2E\ g^+ \rightleftharpoons {}^2E\ g^-$. If a model is assumed in which an equilibrium exists among the classically staggered g^+, g^-, and t conformers, the observed H(3′)–P coupling data in Table II indicate that the combined population of the gauche conformers ($g^+ + g^-$) in the dimers examined is $\simeq$70% and that of the t conformer is 30%. This observation is in essential agreement with the $g^+ + g^-$ populations derived for ApA from ^{13}C–^{31}P couplings based on a three rotamer model (Schleich et al., 1976; Alderfer and Ts'o, 1976). However, extension of the three rotamer model to ^1H–^{31}P and ^{13}C–^{31}P coupling in 3′-AMP results in serious disagreement (Singh et al., 1976). Thus ^{13}C data predict 70% t conformation in 3′-AMP with minimal contribution from g^+ conformer (4%) whereas ^1H data predict 70% $g^+ + g^-$ conformers with significant contribution from g^+ conformers, as revealed from H(2′)–P couplings.

There is general qualitative agreement between the populations of conformers about the C(3′)–O(3′) bond computed from both ^{13}C and ^1H data for monomers and dimers using a two rotamer model of $g^+ \rightleftarrows g^-$ equilibrium. Therefore, it appears that this model is the most consistent with observed NMR data.

(c) P–O(3′), P–O(5′) (ω_1', ω_1). Determination of the conformational preferences about P–O(3′) and O(5′)–P in aqueous solution is difficult because of the lack of appropriate coupling constants. The method employed here is an indirect one in which shift trends are used in conjunction with information from theoretical (Broyde et al., 1974; Kim et al., 1973; Olson, 1973, 1975a–c; Yathindra and Sundaralingam, 1974, 1975) and solid state studies (Rubin et al., 1972; Sussman et al., 1972; Rosenberg et al., 1973; Day et al., 1973; Hingerty et al., 1975). These studies have clearly demonstrated that the values of ω_1'/ω_1 for dinucleoside monophosphates lie in the range expected of a g^-g^- right-handed stack (270°, 270°), a g^+g^+ left-handed stack (90°, 90°), and several extended conformations. In a later section we show that these dimers, in general, exist in aqueous solution as an equilibrium system of stacked and extended conformations. The discussion below is strictly limited toward providing evidence for the presence of the two stacked forms and to a qualitative determination of their relative compositions. In preceding papers (Kondo and Danyluk, 1976; Lee et al., 1976), it was shown that the information which emerges from ring current considerations of base protons (Ts'o et al., 1969; Bangerter and Chan, 1969) cannot differentiate clearly between g^-g^- and g^+g^+ stacks. Our method essentially utilizes the chemical shift trends of H(5′) and H(5″) of the 3′- and 5′-nucleotidyl units upon dimerization to make a possible distinction between these two kinds of stacks.

Evidence for the Presence of Right-Handed Stacks. It is pertinent to note a distinguishing feature in the environment of H(5′) of the 5′-nucleotidyl unit in the two stacked forms. In the g^-g^- conformation, the measured distance between O(2′) of the 3′ fragment and H(5′) of the 5′ unit is 2.5 Å, whereas in the g^+g^+ conformer this distance is 6 Å. Obviously one would expect H(5′) of the 5′ unit to be considerably deshielded by O(2′), should the molecules show a disposition for the g^-g^- stack. Data in Table III indicate a pronounced deshielding of this H(5′) in all the dimers and suggest the presence of right-handed stacks. The H(5′) of the 5′-nucleotidyl unit in GpC undergoes a shift to lower field by 0.234 ppm compared with 0.169 ppm in CpG. This denotes that qualitatively in the matched pairs GpC and CpG, the former has a higher population of g^-g^- stacks. The same is true of the matched pairs ApU and UpA, GpU and UpG. One may conclude that the population of g^-g^- in pu-py dimers is generally greater than in py-pu dimers.

Evidence for the Presence of Left-Handed Stacks. In the g^+g^+ stack, H(5′) and H(5″) of the 3′-nucleotidyl unit reside in the interior of the stack and are in an environment that can substantially influence their chemical shifts. On the other hand, in the g^-g^- stack, the same protons reside on the exterior of the stack in an environment essentially free of the influence of the adjacent base. In the g^+g^+ stack of UpA, for example, H(5′) and H(5″) of Up- reside with cylindrical coordinates z (vertical) of 4 and 3 Å for both nuclei relative to the plane of -pA. Hence in a left-handed stack of UpA, UpG, CpG, and CpA, one would expect the purine moiety to shield H(5′) and H(5″) of Up- and Cp-, the effect being somewhat larger for H(5″). In the g^+g^+ stack of ApU, ApC, GpU, and GpC, the chemical shift of H(5′) and H(5″) of Ap- and Gp- will not be affected by the bases of the 5′-nucleotidyl units because of the small ring current fields of uracil and cytosine moieties (Giessner-Prettre and Pullman, 1970).

Another environmental characteristic of H(5′) and H(5″) of the 3′-nucleotidyl unit in g^+g^+ stacks is their juxtaposition to the 2′- and 3′-hydroxyl group of the ribose ring of the 5′-nucleotidyl unit, i.e., H(5′) and H(5″) are roughly 4 and 5 Å, respectively, distant from these hydroxyl groups. Hence in the g^+g^+ stack one would expect these hydroxyl groups to deshield H(5′) and H(5″) of the 3′-nucleotidyl units, the effect being larger on H(5″).

Thus, two opposing effects control $\delta_{H(5')}$ and $\delta_{H(5'')}$ of the 3′-nucleotidyl units in the g^+g^+ stack: 1. ring current shielding by the 5′ base; this is significant only in the py-pu dimers; 2. deshielding by the 2′ and 3′ hydroxyls of the 5′-nucleotide unit; this is present in both pu-py and py-pu dimers.

The dimerization data in Table III for the Ap- and Gp- H(5′) and H(5″) of ApU, ApC, GpU, and GpC reveal that these protons are deshielded, and further that H(5″) undergoes a larger deshielding. This is a clear indication of the presence of g^+g^+ stacks in aqueous solution for these dimers. In the case of UpA, UpG, CpG, and CpA, the data in Table III reveal that $\delta_{H(5')}$ and $\delta_{H(5'')}$ of Up- and Cp- indeed appear at higher fields, and, in most cases, the effect is larger for H(5″). These data point out the sizeable presence of g^+g^+ stacks in UpA, UpG, CpG, and CpA solutions, and that the shielding effect of the purine is considerably greater than the deshielding effects of the 2′ and 3′ hydroxyls of the 5′ residue.

In short, the dimerization data for H(5′) and H(5″) of the 5′- and 3′-nucleotidyl units can be rationalized best on the grounds that both g^-g^- and g^+g^+ stacks are present in the aqueous solutions for all hetero dimers. In pu-py dimers the population of the g^-g^- species is greater than the g^+g^+ species; also the population of the g^-g^- stacks in pu-py dimers is generally greater than in corresponding matched py-pu dimers.

The argument employed here depends a great deal on the correctness of the assignments of H(5′) and H(5″) signals in the 3′- and 5′-nucleotidyl units. The assignments for the 5′ unit follow those proposed elsewhere (Remin and Shugar, 1972; Davies and Rabczenko, 1975), while those for the 3′ unit concur with the proposal of Son and Guschlbauer (1975). The

observations that on dimerization H(5′) and H(5″) of the 3′-nucleotidyl units shift (1) to higher field with loss of magnetic nonequivalence in py-pu dimers and (2) to lower field with increase in magnetic nonequivalence in pu-py can be rationalized only on the basis of present assignments. It is H(5″) which shows the greatest change because of its closer approach to the 2′ and 3′ hydroxyls and the base of the 5′ unit.

Relative Orientation between Base and Ribose Groups. Numerous previous studies (Lee et al., 1976, and references cited therein) have shown that in aqueous solution the common mono-, oligo-, and polynucleotides exist as an equilibrium mixture of syn and anti orientations with somewhat greater preference for the latter. No satisfactory approach is now available for quantitative evaluation of χ_{CN} or syn/anti ratios, and it is assumed, therefore, that nucleotidyl units of the hetero dimers prefer an anti domain. Despite the above reservation, it is possible to determine relative differences in magnitude of χ_1 and χ_2 as well as the dimerization induced change of χ_{CN}.

Theoretical calculations (Giessner-Prettre and Pullman, 1976) clearly show that $\delta_{H(1')}$ is highly sensitive to torsional variation of χ_{CN}, a reduction of χ_{CN} from 60 → 0° producing upfield shifts ranging from 0.25 to 0.6 ppm for purine and 0.6 to 0.8 ppm for pyrimidine nucleosides. An indication of relative magnitudes of χ_1 and χ_2 can therefore be obtained from a comparison of $\delta_{H(1')}$ trends for 3′ and 5′ nucleotides and dimers with the same base. Inspection of such shift data for 3′ and 5′ mononucleotides (Lee et al., 1976, and Tables II and III) shows H(1′) to be consistently more shielded in the 3′ nucleotide as would be expected for χ_{CN} (3′-) < χ_{CN} (5′-). For both the homo- and heterodipyrimidines H(1′) of the 3′-nucleotidyl unit is about 0.07 ppm upfield relative to H(1′) of the 5′ residue. Again these observations are consistent with $\chi_1 < \chi_2$ in UpU, UpC, CpU, and CpC. Extension of this approach to purine–purine dimers is complicated by the ring-current influence of the adjacent base. A somewhat more favorable situation obtains for purine nucleotides of matched pu-py, py-pu pairs since ring current effects of adjacent pyrimidines on H(1′) are minimal in these systems. Comparison of data in Table I shows that for the pairs, ApU/UpA, ApC/CpA, GpC/CpG, $\delta_{H(1')}$ of pu- appears at a higher field (0.05–0.21 ppm) than in -pu implying that χ_1 (pu-) < χ_2 (-pu) for these dimers.

Similar arguments can be used to assess χ_{CN} trends on dimerization, but, as noted above, the $\Delta\delta_{H(1')}$(dimer) values for purine residues are more representative of glycosidic torsion angle changes than are the changes for pyrimidine residues. Analysis of the data in Table III shows that in all cases H(1′) and H(2′) of pu- nucleotides are more shielded in the dimers consistent with a decrease in χ_{CN}. Further confirmation of this conclusion is given by temperature measurements which show a deshielding of H(1′) and H(2′) of Ap- and -pG for ApC and CpG with increase in temperature indicating that temperature-induced destacking causes increases in values of χ_1 and χ_2. It should be noted that although the anomeric proton shifts for pyrimidine nucleotidyl units are less definitive, the occurrence of χ_{CN} changes can be inferred on the basis of the existence of a coupled equilibrium between ribose conformation and χ_{CN} (vide infra).

Base–Base Interaction. Extensive investigations in aqueous solutions at biological pH and temperature have shown that dinucleoside monophosphates exist as equilibrium mixtures of extended (unstacked) and stacked conformers and that elevation of temperature causes an increase in the population of the extended forms at the expense of the stacked conformers (Ts'o et al., 1969; Chan and Nelson, 1969; Hruska and Danyluk, 1968). These conclusions were reached by examining shift data for base and anomeric protons. From the dimerization data for H(5) (Table III), one can immediately observe that a range of stacking exists for the heterodimers from a maximum in GpC ($\Delta\delta_{H(5)}$ = 0.476) to a minimum in UpG ($\Delta\delta_{H(5)}$ = 0.122). In addition the dimers can be separated into two classes: the pu-py with $\Delta\delta_{H(5)}$ = 0.23–0.48 Hz and py-pu with $\Delta\delta_{H(5)}$ = 0.12–0.28 ppm.

The two classes are listed separately and in decreasing order of $\Delta\delta_{H(5)}$ in Table VI: i.e., GpC > ApC > ApU > GpU and CpA > CpG > UpA > UpG. It appears on this basis that the population of stacked species in pu-py is significantly greater than in py-pu. However, it is possible that the population of stacked species, and sugar–base and backbone conformations are very similar for pu-py and py-pu dimers and the observed difference in the $\Delta\delta_{H(5)}$ values between the two classes is merely a reflection of the difference in the degree of overlap between the bases. In fact, this argument has been advocated by Bangerter and Chan (1969) who concluded that ApC and CpA have nearly identical stacked populations and geometries, the only difference being in the degree of overlap. Their argument was based on similarities in the H(1′)–H(2′) coupling constants in ApC and CpA. However, these couplings were obtained from simple first-order analysis of complex 60- or 100-MHz spectra and are evidently in error. As discussed earlier in this work, $J_{H(1')H(2')}$ is consistently smaller in the pu-py class of dimers, while ΔJ, $(J_{H(1')H(2')})_{monomer} - (J_{H(1')H(2')})_{dimer}$, is smaller in the py-pu dimers. The ΔJ values are listed in Table VI in decreasing order. As can be seen, there is a striking correlation between ΔJ and $\Delta\delta_{H(5)}$. As ΔJ decreases so does $\Delta\delta_{H(5)}$ with a maximum value in GpC and minimum in UpG. Since ΔJ is a monitor of stacking (Lee et al., 1976), $\Delta\delta_{H(5)}$ does not represent differences in the degree of overlap between the bases in the two classes of dimers, as has been argued by Bangerter and Chan (1969). This must mean that a clear difference exists in the stacking properties of pu-py and py-pu dimers. This difference may be related to: (1) overall differences in the population distribution of stacked and extended species; (2) population differences between right- and left-handed stacks, i.e., g^-g^- and g^+g^+.

Without attempting to distinguish between the two stacked and extended forms, one may obtain quantitative information about the population of stacked and nonstacked forms from the ribose coupling constants using the method discussed extensively elsewhere (Lee et al., 1976; Altona, 1975). The computed populations are summarized in Table V. The percent stacking ranges from 45% in GpC to 10% in UpG.

One obvious advantage of using the coupling constants to estimate stacking is that it allows a direct comparison to be made between the different classes of dimers: pu-py, py-pu, pu-pu, and py-py. The values for the homodimers range from 25 to 38% for pu-pu vs. 10–35% for py-py (Lee et al., 1976). These values fall within the range represented by the heterodimers (Table V). The overall sequence for the stacking proclivities of bases in dinucleoside monophosphates is as follows: py-pu ≤ py-py < pu-pu < pu-py.

Another index of stacking interactions is provided by the magnetic nonequivalence of 5′,5″ protons of the 5′-nucleotidyl fragment (lee et al., 1976) relative to that in the monomer, i.e., $\Delta = |(\delta_{5'} - \delta_{5''})_{dimer} - (\delta_{5'} - \delta_{5''})_{monomer}|$. The Δ's are listed in Table III and again in Table VI for the purpose of comparison with the other stacking indices. Again a direct correlation is observed among the ΔJ, $\Delta\delta_{H(5)}$, and Δ. There is one significant discrepancy and that is for the dimer ApA. According to ΔJ and $\Delta\delta_{H(5)}$ values, GpC has the maximum

population of stacked conformers; but with Δ as a monitor of stacking (Lee et al., 1976), ApA shows the maximum percentage in stacking. This difference arises because $\Delta\delta_{H(5)}$ and ΔJ monitor primarily the total population of stacked conformers ($g^-g^- + g^+g^+$) and Δ monitors significantly the fractional population of the g^-g^- stacks.

In the g^+g^+ stacks, H(5′) and H(5″) of the 5′-nucleotidyl unit reside on the exterior of the stack in an environment which has minimal influence on the chemical shifts of H(5′) and H(5″) of the 5′ unit; i.e., the conclusion is that the g^-g^- population in ApA is more than the g^-g^- population in GpC even though the combined $g^-g^- + g^+g^+$ populations in GpC are greater compared to ApA. Conversely, the g^+g^+ population in GpC is greater than that in ApA. The major point to be reiterated is our contention that a base sequence effect exists on the stacking of bases in the dimers contrary to the claims of Bangerter and Chan (1969). Our NMR findings are in line with results from such diverse methods as fluorescence and phosphorescence (Eisinger et al., 1966), electron spin resonance (Gueron et al., 1966), laser-Raman (Thomas, 1975) UV absorbance (Warshaw and Tinoco, 1966), and ORD (Davis and Tinoco, 1968) measurements.

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