Nuclear Magnetic Resonance Studies of 2′- and 3′-Ribonucleotide Structures in Solution†

David B. Davies‡ and Steven S. Danyluk*

ABSTRACT: A systematic 220-MHz proton nuclear magnetic resonance (nmr) study has been made of all common purine and pyrimidine 2′(3′)-ribonucleotides in D_2O solutions at 20 ± 2°. Spectra for the entire series were measured under similar conditions of concentration, temperature, and ionic strength, thereby facilitating intercomparisons of spectral properties. Spectral assignments were accomplished with the aid of selected ^{31}P-^{1}H decoupling experiments and accurate values of nmr parameters were derived by simulation-iteration procedures. A detailed analysis of the coupling constants and chemical shifts permitted a determination of conformational properties for ribose rings, exocyclic carbinol and phosphate groups, and the orientation of base-ribose rings. Following procedures described elsewhere, an evaluation was made of ribose ring pseudorotational parameters for each 2′(3′)-nucleotide. The results show that both the degree of pucker and pseudorotational angle vary only slightly throughout the entire series of molecules, and lie within the ranges found in the crystalline state. Furthermore, the ribose rings are in rapid equilibrium between N type [C(3′)-endo, C(2′)-exo] and S type [C(2′)-endo, C(3′)-exo] conformers, N ⇄ S, with an S type conformer favored in purine 2′(3′)-ribonucleotides (~60:40) while pyrimidines exhibit approximately equal compositions. Thus, the phosphate location on the ring has less of an effect on ring properties than the nature of the base ring. An analysis is also reported of rotamer equilibria about C(4′)-C(5′), C(2′)-O(2′), and C(3′)-O(3′) bonds. For the former the nmr coupling constant data are consistent with a predominant gg rotamer (~70%) with gt and tg rotamers populated to the extent of ~20 and ~10%, respectively. No correlation of the type seen for 5′-nucleotides appears to exist between C(4′)-C(5′) gg population and ribose ring equilibrium composition. For 2′-nucleotides the ^{31}P-H(2′) coupling data indicate a preferred C(3′)g, C(1′)t conformer about C(2′)-O(2′) in agreement with ^{13}C nmr results. A less definitive rotamer analysis follows from observed $J_{^{31}P,H(3')}$ values, but when these results are combined with relevant chemical shift data for deoxynucleosides and nucleotides the evidence strongly points to essentially free rotation and approximately equal rotamer populations about C(3′)-O(3′). Chemical shift differences between purine and pyrimidine 2′(3′)-ribonucleotides are qualitatively accounted for by "in-plane" purine diamagnetic anisotropy effects. Also, the greater magnitude for purine deshieldings in 2′(3′)-nucleotides relative to 5′-nucleotides is explained by a more favored syn:anti ratio in the former in line with recent nuclear Overhauser results. Comparison of the present work with earlier results for 5′-nucleotides reveals a remarkable consistency in almost all of the conformational features for the basic nucleotidyl unit throughout the entire range of mononucleotides. An important exception is the difference in rotamer behavior for exocyclic phosphate groups in 5′- and 3′-nucleotides. The greater rotational freedom in the latter has important consequences for conformational properties of dinucleotides and higher nucleic acid polymers.

Ribo- and deoxyribonucleotides with a phosphate group at either 5′ or 3′ positions are the simplest nucleotidyl building blocks of RNA and DNA. For this reason quantitative structural and conformational data are vital for both classes of molecules as a starting point for structure/conformation determinations of higher chain-length nucleic acids in solution. Additionally, accurate solution data permit an intercomparison with crystal structure results, thereby identifying key repetitive structural features in nucleic acids in general. A preceding paper (Davies and Danyluk, 1974) reported an extensive and systematic study of proton nuclear magnetic resonance (nmr) spectra for all common naturally occurring 5′-ribo- and deoxyribonucleotides. Parameters derived from the spectra served as a basis for the calculation of furanose ring pseudorotational parameters and exocyclic group rotamer populations for each class of nucleotide. These calculations along with consideration of chemical shift trends permitted a definition of overall conformational features and systematic conformational trends. A surprising result revealed by this work was the relative constancy in structural properties for 5′-ribo- and deoxyribonucleotides in aqueous solution.

We now report results of a comparable study of nmr spectra and conformational calculations for 2′- and 3′-ribonucleotides. Proton and ^{13}C nmr spectra have been reported previously for several of the nucleotides (Jardetzky, 1962; Kreishman and Chan, 1971; Schleich *et al.*, 1972; Tran-Dinh Son *et al.*, 1972; Tran-Dinh Son and Chachaty, 1973; Mantsch and Smith, 1972; Smith *et al.*, 1973). In order to provide a consistent data base for conformational calculations, proton spectra were measured at 220 MHz for all of the common 2′- and 3′-nucleotides in aqueous solution at fixed solvent and concentration conditions. Several of the nucleotides were studied in both salt and free acid form to assess the effect of ionization state upon ribose ring conformation. A detailed calculation of pseudorotational parameters for ribose rings and exocyclic rotamer populations was

† From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439. *Received August 5, 1974.* This work was supported by the U. S. Atomic Energy Commission. Paper two of a series on nmr studies of nucleic acids in solution.

‡ Present address: Department of Chemistry, Birkbeck College, Malet Street, London WC1E 7HX.

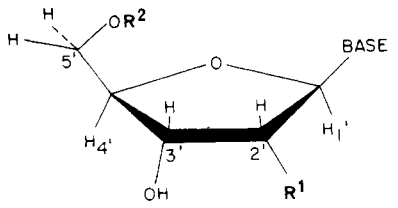


FIGURE 1: Numbering scheme for ribose ring. R^1 = OH = ribonucleoside; $R^1 = (PO_3)^-$ = nucleotide; R^2 = H = nucleoside; $R^2 = (PO_3)^-$ = nucleotide.

made following procedures described in earlier work (Blackburn *et al.*, 1970; Altona and Sundaralingam, 1972, 1973; Davies and Danyluk, 1974). Particular interest was focused on the effect of phosphate group position upon ribose conformation and upon regularities in conformational features throughout the series. As with the 5′-nucleotides the evidence points to surprisingly constant conformational properties for 2′- and 3′-ribonucleotides.

Experimental Section

Materials. The nucleotides (highest purity) were purchased from Sigma Chemical Co. and were used without further purification. D_2O (99.8%) used for preliminary lyophilization was obtained from U.S.A.E.C. and D_2O (100.0%) employed subsequently for preparation of solutions for nmr analysis was purchased from Diaprep. The sodium salt of 3-trimethylsilylpropionic-*2,3,3,3-d*$_4$ acid (TSP) was purchased from Merck, Sharp and Dohme.

Preparation of Samples. Commercial samples of nucleotides contain some residual hydrate H_2O, and exchangeable phosphate acid protons, base-ring protons, and hydroxyl groups which contribute to a residual HDO signal in D_2O solutions. Nucleotide samples were accordingly lyophilized several times from 98% D_2O to minimize the residual HDO signal and then dissolved in 100.0% D_2O with a final solution concentration of 0.1 M.

Measurement of Spectra. Proton spectra were measured with a Varian HR 220 spectrometer operating at a probe temperature of ~20 ± 2°. All of the signal positions were measured relative to internal TSP and calibrations were made by the audio side-band method using a Hewlett-Packard 4204A oscillator calibrated in turn with a Hewlett-Packard 5245L frequency counter. Signal positions measured in this manner are accurate to 0.002 ppm. Where necessary signal assignments were confirmed by appropriate ^{1}H-[^{31}P] spin-decoupling experiments using a Rhode and Schwartz XV5D frequency generator with X10 multiplier operating at approximately 89 MHz. Spectral parameters (δ and J values) were determined using a combination of simulation and iterative analysis programs. Initial sets of parameters were derived by simulating best visual fit spectra using a Varian 6-spin nmr simulation program. These parameters served as input to an NMREN-NMRIT program which gave a further refinement to final chemical shifts and coupling constants.

Results

2′-Ribonucleotides. An illustration of the chemical structure and numbering scheme followed for 2′- and 3′-ribonucleotides is shown in Figure 1. Analyses of the spectra were accomplished as described in the following. Figure 2 shows the 220-MHz spectrum for ribose ring protons of CMP-2′ [1] (lithium salt) in D_2O. The spectrum is nearly first order for most of the multiplets and assignment of the signals follows readily from a consideration of multiplet splitting patterns and chemical shifts. The negatively charged phosphate group exerts a sizable deshielding on neighboring protons with the greatest effect observed for the C(2′) proton signal observed at 4.63 ppm. The C(2′) proton signal appears as a pseudoquartet arising from coupling to H(1′), H(3′), and ^{31}P (confirmed by ^{1}H-[^{31}P] decoupling). A somewhat larger chemical shift difference is observed between the two C(5′) protons (δ ~3.85 ppm, Figure 2) than in corresponding 5′-nucleotides (Davies and Danyluk, 1974) thereby permitting a determination of all relevant coupling constants in the former.[2] The spectra for AMP-2′, GMP-2′, and UMP-2′ are similar to that for CMP-2′ and signal assignments and analysis followed procedures as above. The spectrum of GMP-2′ closely resembles that already published (Tran-Dinh Son *et al.*, 1972) though discrepancies in ribose ring chemical shifts of about 0.435 ppm are found as the previous values were measured with respect to an external Me_4Si capillary for which no bulk magnetic susceptibility correction was made. The spectrum of AMP-2′ is also similar to that observed by Tran-Dinh Son and Chachaty (1973) and, in this case, the chemical shifts show smaller variations (0.02–0.06 ppm) as the values were reported with respect to internal DSS (2,2-dimethyl-2-silapentane-5-sulfonate) used as reference standard. A summary of chemical shifts and coupling constants for the 2′-nucleotides measured in this work is given in Tables I and II, respectively. It should be noted from Table I that the chemical shifts of purine and pyrimidine 2′-ribonucleotides are similar within each class of molecules. Deshielding of all the ribose ring proton signals is observed for purine compared to pyrimidine derivatives (H(5′)(5″) 0.03 to H(2′) 0.413 ppm).

3′-Ribonucleotides. The spectra of AMP-3′, GMP-3′, and UMP-3′ were measured as their sodium salts and CMP-3′ was measured as the lithium salt and in the free acid form.

The 220-MHz proton spectrum of CMP-3′(H^+) is illustrated in Figure 3. Only small variations are found in the spectra of the lithium salt and acid form of CMP-3′. An assignment of C(1′), C(4′), and two C(5′) proton signals[2] follows directly from their relative chemical shifts and spin coupling patterns; the remaining multiplets at 4.4–4.6 ppm, corresponding to two protons, are due to H(2′) and H(3′). These in turn are assigned by ^{31}P decoupling experiments, *cf.* upper and lower spectra in Figure 3. It should be noted that H(3′) is downfield compared to H(2′) (opposite to purine derivatives). This point is relevant to the assignment of signals for more complex dinucleotides. A small, long-range ^{31}P–H coupling (<0.5 Hz) can be observed to the H(4′) signal by ^{31}P spin-decoupling experiments. The assignment of the spectrum was confirmed by iterative analysis and a plot of the final calculated spectrum is shown in Figure 3. The spectrum of UMP-3′ was very similar to CMP-3′ and

[1] Abbreviations used are: the 2′-ribonucleotides of adenine (AMP-2′), guanine (GMP-2′), uracil (UMP-2′), and cytosine (CMP-2′); the 3′-ribonucleotides of adenine (AMP-3′), guanine (GMP-3′), uracil (UMP-3′), and cytosine (CMP-3′); 3′-deoxyribonucleotides of adenine (dAMP-3′), guanine (dGMP-3′), cytosine (dCMP-3′), and thymine (TMP-3′).

[2] The assignment of the C(5′) proton signals to individual protons follows on the basis of selective phosphate shielding effects (Remin and Shugar, 1972).

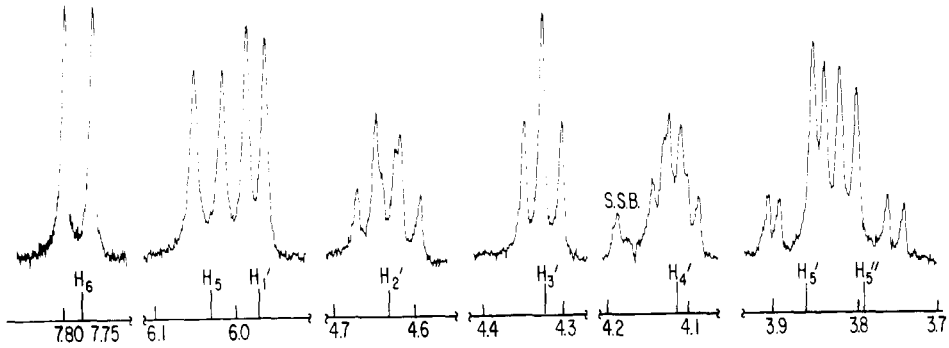


FIGURE 2: The 220-MHz proton magnetic resonance spectrum of 0.1 M CMP-2′ (lithium salt) in D_2O at 20°. Chemical shifts are in ppm relative to internal TSP.

Table I: Proton Chemical Shifts of 2′- and 3′-Ribonucleoside Monophosphates in Aqueous Solution.[a]

Nucleotide	Salt			H_1'	H_2'	H_3'	H_4'	H_5'	H_5''
Purines		H_8	H_2						
AMP-2′	Na^+	8.326	8.117	6.127	5.036	4.572	4.300	3.913	3.840
GMP-2′	Na^+	7.990		6.003	5.090	4.570	4.243	3.871	3.804
AMP-3′	Na^+	8.337	8.151	6.095	4.827	4.730	4.464	3.934	3.934
AMP-3′[b]	H^+	8.326	8.123	6.090	4.872	4.806	4.481	3.947	3.890
GMP-3′	Na^+	8.015		5.929	4.789	4.743	4.387	3.916	3.888
Pyrimidines		H_6	H_5						
UMP-2′	Na^+	7.840	5.890	5.968	4.668	4.350	4.126	3.861	3.793
CMP-2′	Li^+	7.776	6.031	5.967	4.631	4.322	4.118	3.864	3.790
UMP-3′	Na^+	7.890	5.890	5.927	4.395	4.484	4.227	3.893	3.852
CMP-3′	H^+	7.926	6.081	5.918	4.409	4.531	4.277	3.936	3.845
CMP-3′	Li^+	7.874	6.062	5.947	4.386	4.503	4.235	3.930	3.883

[a] Proton chemical shifts measured from TSP as internal reference in 0.1 M D_2O solutions at 291° to an accuracy of ±0.002 ppm. [b] Measured at 70°.

Table II: Spin Coupling Constants of 2′- and 3′-Ribonucleoside Monophosphates in Aqueous Solution.[a]

Nucleotide	Salt	1′,2′	2′,3′	3′,4′	4′,5′	4′,5″	5′,5″	2′,P	3′,P	4′,P
AMP-2′	Na^+	6.0	5.2	3.0	2.6	3.6	−12.9	6.9		
GMP-2′	Na^+	5.2	5.15	3.9	2.8	4.3	−12.8	7.2		
UMP-2′	Na^+	5.4	5.6	4.4	2.9	4.7	−12.8	6.8		
CMP-2′	Li^+	5.0	5.2	5.0	2.8	4.7	−12.6	7.0		
AMP-3′	Na^+	6.3	5.2	3.2	3.0	3.0	−12.7	<0.5	8.0	
AMP-3′[b]	H^+	6.2	5.3	3.2	2.8	3.8	−12.8	1.0	8.6	~0.5
GMP-3′	Na^+	5.2	5.15	3.6	2.6	4.0	−12.7	<0.5	7.3	
UMP-3′	Na^+	4.2	4.8	5.4	2.8	3.8	−12.8		8.0	
CMP-3′	H^+	4.3	5.0	5.4	2.6	4.0	−12.8		8.2	<0.5
CMP-3′	Li^+	4.0	5.1	5.8	2.7	4.5	−13.0		7.5	

[a] Spin coupling constants determined to an accuracy of ±0.1 Hz. [b] Measured at 70°.

the derived parameters are given in Tables I and II. The parameters derived for UMP-3′ are very close to those observed previously (Schleich *et al.*, 1972) with only minor chemical shift differences.

The 220-MHz proton spectrum of the ribose ring protons of AMP-3′ is illustrated in Figure 4. The signals for the two base ring protons, H(2) (8.117 ppm) and H(8) (8.326 ppm), are not shown. Assignment of the C(1′), C(4′), and two C(5′) proton signals[2] follows directly from their relative chemical shifts and spin coupling patterns. The complex multiplet between 4.8 and 4.9 ppm, corresponding to two protons, results from the C(2′) and C(3′) proton signals. Phosphorus decoupling experiments allow a further resolution of the multiplets as illustrated in Figure 4 (upper spectrum). Irradiation at ~89 MHz produces a sharpening of the upfield multiplet to a pseudotriplet and collapses the multiplet at low field to a four-line pattern. Comparison of coupled and decoupled spectra permits an assignment of the multiplet at 4.88 ppm to H(2′) and the signal at 4.05 ppm to H(3′) with $J_{^{31}P,H(3')} = 8.6$ Hz. Long range ^{31}P–H cou-

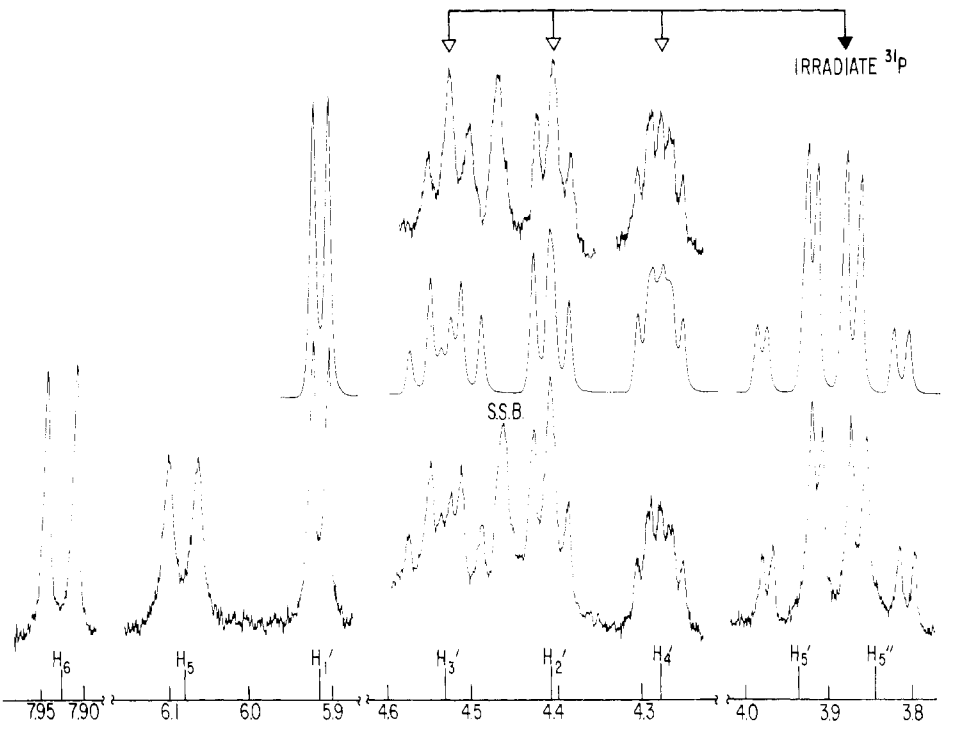


FIGURE 3: Lower spectrum: 220-MHz pmr spectrum of CMP-3′ (free acid) in D_2O at 20° (S.S.B. = spinning side band of HOD signal). Chemical shifts are in ppm relative to internal TSP. Middle spectrum: Computer simulated spectrum of ribose ring protons using values in Tables I and II. Upper spectrum: ^{31}P decoupled 220-MHz pmr spectrum of CMP-3′.

plings[3] are also readily observable with H(2′), $^4J_{^{31}P,H(2')} \sim 1.0$ Hz, and $^4J_{^{31}P,H(4')} \sim 0.5$ Hz. Assignment of the spectrum was confirmed by iterative analysis and a plot of the final calculated spectrum is shown in Figure 4. The spectrum of AMP-3′ is similar to that observed by Tran-Dinh Son and Chachaty (1973). Small variations in chemical shifts are found [0.07 ppm H(3′) − 0.12 ppm H(2′)] similar to those noted for AMP-2′ by the same workers. The spectrum of GMP-3′ is very close to that of AMP-3′ and the derived parameters are given in Tables I and II.

The summary of chemical shifts for the 3′-nucleotides measured in this work (Table I) indicates that the values within purine and pyrimidine series are similar to each other but that deshielding of all ribose ring proton signals is observed for purine compared to pyrimidine derivatives [0.012 ppm H(5′) to 0.418 ppm H(2′)] similar to that found for 2′-ribonucleotides. The combination of purine ring and phosphate group deshielding effects are responsible for H(2′) being downfield compared to H(3′) for the purine 3′-ribonucleotides whereas the reverse situation is found for pyrimidine 3′-ribonucleotides.

Discussion

As with 5′-nucleotides, the conformational properties for 2′- and 3′-nucleotides are determined by several key features, namely, ribose ring conformation, exocyclic group orientations (about C(4′)-C(5′) and C(2′)(3′)-O(2′)(3′) bonds) and base–ribose ring orientations. In each instance conformational information can be derived from appropriate coupling constant and chemical shift data following procedures analogous to those developed for 5′-nucleotides.

1. Ribose Ring Conformation: Pseudorotational Analysis. In comparison with nucleosides and 5′-nucleotides relatively few crystal structure determinations have been reported for either 2′- or 3′-ribonucleotides. A recent compilation of X-ray diffraction data (Altona and Sundaralingam, 1973, abbreviated AS) shows that both N type (C(3′)-endo) and S type (C(2′)-endo) conformations exist for 3′-nucleotides, with pseudorotational angles P, and degree of pucker τ_m, falling in the ranges encompassing the majority

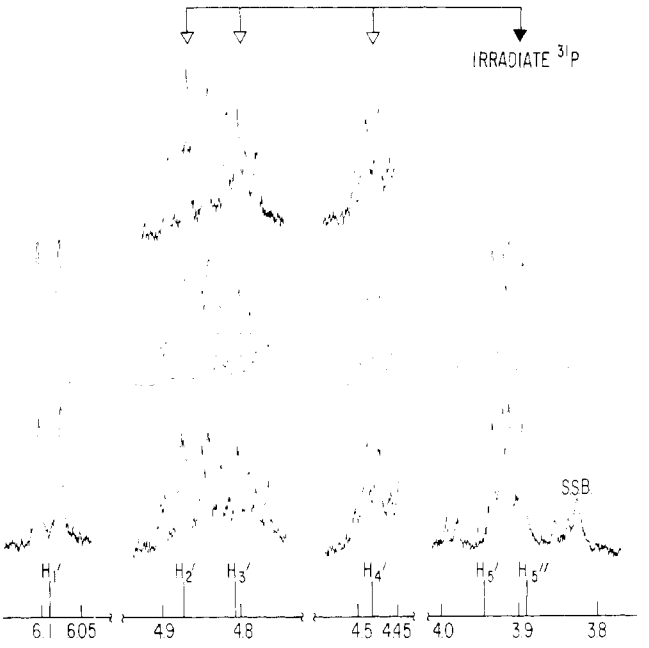


FIGURE 4: Lower spectrum: 220-MHz pmr spectrum of AMP-3′ in D_2O at 70° (S.S.B. = spinning side band of HOD signals); chemical shifts are in ppm relative to internal TSP. Middle spectrum: Computer simulated spectrum using values in Tables I and II. Upper spectrum: ^{31}P decoupled 220-MHz pmr spectrum of AMP-3′.

[3] Decoupling experiments designed to establish limits for the magnitude of 4-bond and 5-bond couplings showed that in all cases except $^4J_{^{31}P,H(2')}$ and $^4J_{^{31}P,H(4')}$ the couplings were less than 0.2 Hz.

Table III: Pseudorotational Parameters and Equilibrium Compositions for the Purine and Pyrimidine 2′- and 3′-Ribonucleotides (Salts) in Aqueous Solution Using an Average $J_{2',3'} = 5.2$ Hz.

Nucleotide	P_N (deg)	τ_N	P_S (deg)	τ_S	${}^{N}J_{1',2'} = {}^{S}J_{3',4'}$	${}^{N}J_{3',4'} = {}^{S}J_{1',2'}$	%N	K_{eq}	$K_{eq}{}^{f}$
UMP-2′	20	37	163	39	0.1	9.7	45	1.22	1.23
CMP-2′	23	38	160	40	0.15	9.85	50	1.00	1.00
UMP-3′	17	37	166	39	0.05	9.55	56	0.79	0.78
CMP-3′	20	36	163	39	0.1	9.7	59	0.69	0.69
Mean	20 (±2)	37 (±1)	163 (±2)	39 (±1)			53 (±5)		
AMP-2′	7	35	175	38	0	9.0	33	2.03	2.00
GMP-2′	9	35	174	38	0	9.1	43	1.32	1.33
AMP-3′	14	35	169	38	0.05	9.35	34	1.94	1.94
GMP-3′	4	35	178	38	0	8.8	41	1.43	1.44
Mean	9 (±3)	35 (±1)	174 (±2)	38 (±1)			38 (±4)		
AMP-2′[a]	14	36	169	39	0.05	9.35	36	1.78	1.76
AMP-2′[b]	10	35	172	38	0	9.2	38	1.63	1.63
AMP-3′[b]	6	35	175	38	0	9.0	36	1.78	1.65
GMP-2′ (pD 7.4)[c]	21	37	161	40	0.15	9.75	45	1.22	1.20
GMP-2′ (pD 1.2)[c]	29	40	153	43	0.35	10.15	54	0.85	0.88
UMP-3′[d]					No fit				1.00
UMP-3′ (23°)[e]	27	39	156	42	0.25	10.05	54	0.85	0.87
UMP-3′ (88°)[e]	29	40	153	40	0.35	10.15	53	0.89	0.91
β-ψ-UMP-3′ (23°)[e]	33	42	149	44	0.50	10.3	51	0.96	0.96
β-ψ-UMP-3′ (75°)[e]	36	43	147	45	0.60	10.5	50	1.00	0.98

[a] Jardetzky (1962). [b] Tran-Dinh Son and Chachaty (1973). [c] Tran-Dinh Son *et al.* (1972). [d] Kreishman and Chan (1971). [e] Schleich *et al.* (1972). [f] Calculated using relation $K_{eq} = J_{1'2'}/J_{3'4'}$ (Davies and Danyluk, 1974).

of nucleotide derivatives. Accordingly, $\bar{P}$ and $\bar{\tau}_m$ values calculated for all derivatives and summarized in an earlier paper (Table III, Davies and Danyluk, 1974) can be used in comparison of conformational properties for 2′(3′)-nucleotides in crystal and solution states.

A necessary condition for an AS analysis of nmr data for ribose rings of nucleotides is a constancy of $J_{2'3'}$ and $(J_{1'2'} + J_{3'4'})$ in a series of molecules. The magnitudes of appropriate J's in Table II show that these conditions are fulfilled satisfactorily for 2′(3′) nucleotides with $\bar{J}_{2'3'} = 5.2 \pm 0.2$ Hz and $(\bar{J}_{1'2'} + \bar{J}_{3'4'}) = 9.5 \pm 0.3$ Hz. The value for $\bar{J}_{2'3'}$ is close to that for unsubstituted ribose rings in nucleosides, 5.1 Hz (AS, 1973), and for 5′-nucleotides, 4.9 ± 0.1 Hz (Davies and Danyluk, 1974). It was shown in the latter work that a variation in $J_{2'3'}$ of up to ±0.2 Hz produces only slight changes in pseudorotational parameters. It can be seen that substitution of a 2′(3′)-hydroxyl by a phosphate does not alter $\bar{J}_{2'3'}$ to any significant extent, suggesting a minimal inductive effect from the latter. This conclusion was used in the analysis of the conformational properties of the exocyclic phosphate group of 5′-nucleotides (Davies and Danyluk, 1974). The value for $(\bar{J}_{1'2'} + \bar{J}_{3'4'})$ differs by 0.6 Hz from the reported average for various nucleosides, 10.1 Hz (AS, 1973), but is within experimental error of that for 5′-nucleotides, 9.3 ± 0.3 Hz.

Following procedures described previously (AS, 1973, Davies and Danyluk, 1974) $\bar{J}_{2'3'}$ and $(\bar{J}_{1'2'} + \bar{J}_{3'4'})$ can be related to constants of the Karplus expression $J(\mathrm{H,H}) = A\cos^2\theta + B\cos\theta$ by

$$\bar{J}_{2'3'} = 5.2 = 0.57A - 0.75B \quad (1)$$

$$\bar{J}_{1'2'} + \bar{J}_{3'4'} = 9.5 = 0.86A + 0.93B \quad (2)$$

from which it follows that $A = 10.2$ and $B = -0.8$. These values differ somewhat from constants for nucleosides, $A = 10.5$, $B = -1.2$ (AS, 1973) and 5′ nucleotides, $A = 9.8$, $B = -0.9$ (Davies and Danyluk, 1974) and are reflected in small changes of the curves used to evaluate pseudorotational parameters P, τ_m. The latter were interpolated from a set of curves analogous to those calculated for 5′-nucleotides (Figure 6, Davies and Danyluk, 1974). A summary of all the final pseudorotational parameters for 2′(3′)-ribonucleotides is given in Table III.

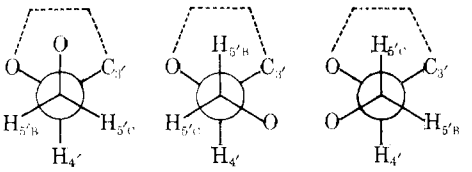
Degree of Pucker, τ_m. The results in Table III indicate that the degree of pucker in 2′(3′)-ribonucleotides is apparently independent of purine or pyrimidine base in both N and S conformers. Although the data also show ${}^{N}\tau_m$ (average), 36 ± 1, to be less than ${}^{S}\tau_m$ (average), 39 ± 1, this difference reflects the method of analysis using a composite Karplus relation for each molecular fragment rather than any real conformational effect (Davies and Danyluk, 1974). Somewhat higher (${}^{N}\tau_m$ and ${}^{S}\tau_m$) values are calculated using data of other workers but these can be attributed to variations in reported coupling constant magnitudes for $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$. The degree of pucker is essentially the same as in 5′-ribo- and deoxyribonucleotides and is evidently not affected by changes in phosphate position on the ring. This similarity, however, may be due to the relative insensitivity of τ_m to ribose ring couplings (Figure 6, Davies and Danyluk, 1974).

Pseudorotational Angle, P. The pseudorotational angles determined from nmr parameters of this study lie in ranges of 4–23° for P_N, and 160–178° for P_S, and are within values found for nucleosides and nucleotides in the crystalline state. When nmr parameters of other workers are included, P_N and P_S show a further spread to 4–36° and 147–178°, respectively (Table III). Two distinct groups of P values are noted for purine and pyrimidine nucleotides

Table IV: Calculated Populations of the Three Classical Rotamers of the $-CH_2OH$ Group.[a-c]

Nucleotide	ρ_I (gg)	ρ_{II} (tg)	ρ_{III} (gt)
AMP-2′	0.78	0.06	0.16
GMP-2′	0.69	0.08	0.23
AMP-3′ (Na^+)	0.80		
AMP-3′ (H^+)	0.74	0.08	0.18
GMP-3′	0.74	0.06	0.20
UMP-2′	0.64	0.09	0.27
CMP-2′	0.65	0.08	0.27
UMP-3′	0.74	0.08	0.18
CMP-3′	0.68	0.07	0.25

[a] Calculations made using equations summarized in Table VII, Davies and Danyluk (1974). [b] Assignment of C(5′) signals according to Remin and Shugar (1972). [c] Diagrammatic representation of C(4′)-C(5′) bond staggered conformations.



I II III

within each conformer type.[4] Thus for N conformers both 2′ and 3′ pyrimidine nucleotides have a P_N close to the mean of 20 ± 3°, while purine 2′(3′) nucleotides lie near a mean of 9 ± 3°. A similar grouping of purine and pyrimidine pseudorotational angles occurs for the S conformer, with P_S(purine) > P_S(pyrimidine). These observations differ from crystallographic findings which show $\bar{P}_S$(purine) = 158 ± 7° < $\bar{P}_S$(pyrimidine) = 166 ± 7°, and $\bar{P}_N$(purine) ≈ $\bar{P}_N$(pyrimidine) ≈ 10°. As most of the crystal analyses were for unsubstituted ribose rings, differences in crystal and solution trends might be attributed to the presence of a phosphate group. Yet, this possibility is questionable because there is no detectable effect of phosphate position, with 2′ and 3′ derivatives showing the same τ and P values for a given purine or pyrimidine base. Further complicating the picture is an absence of base-ring dependence in 5′-nucleotides. It would appear, then, that the pseudorotational angle is dependent in some way upon the base ring in 2′(3′)-ribonucleotides, but the nature of such a correlation is not evident from the data.

Equilibrium compositions were calculated by the full AS pseudorotational analysis and by a simple "direct" method described for 5′-nucleotides (Davies and Danyluk, 1974).

$$J_{1'2'} = (1 - X_N)9.5 \quad (3)$$

$$J_{3'4'} = 9.5X_N \quad (4)$$

Equations 3 and 4, where X_N is the N conformer mole fraction, were used for direct calculation and were derived following arguments outlined in the 5′-nucleotide work. Both sets of calculated equilibrium constants are compiled in Table III and excellent agreement is obtained between the two approaches. Because of its simplicity the direct approach has considerable appeal, but its use is restricted to closely related families of molecules.

From the equilibrium composition in Table III, it follows that phosphate group location in the ring has much less impact upon conformer population than the nature of the base ring. Thus, a slight preference (52 ± 5) is shown for an N conformer in 2′ and 3′ pyrimidine nucleotides, while the S conformer is decidedly more favored in 2′(3′) purines (62 ± 4). In contrast, the S form is preferred in both purine and pyrimidine 5′-ribo- and deoxyribonucleotides (43 ± 3 and 30 ± 1), with no detectable base effect.

Analysis of earlier nmr data leads to ribose conformer populations in line with the present results (Table III). UMP-3′ and β-ψ-UMP-3′ show identical N populations despite a change in glycosidic bonding arrangements.[5] For GMP-2′, a decrease in pD from 7.5 to 1.2 produces a detectable shift from S to N conformer suggesting that changes in ionization state of a base ring are transmitted to conformational differences in the ribose group.

2. Conformation of Exocyclic $-CH_2OH$ Group. Previous analyses (Hruska *et al.*, 1970; Grey *et al.*, 1971; Blackburn *et al.*, 1970) of $J_{4'5'}$ and $J_{4'5''}$ couplings for exocyclic groups of nucleosides have established the existence of rapid rotation (on the nmr time-scale) about the C(4′)-C(5′) bond. The coupling constants can be interpreted in terms of three classical staggered rotamers, gg, tg, gt (Table IV) with a gg conformer preferred in all of the nucleosides. The calculation of the gg conformer population requires the sum of $J_{4'5'}$ and $J_{4'5''}$ whereas calculations of gt and tg populations depend on an unequivocal assignment of the two exocyclic methylene protons. In previous rotamer calculations for nucleosides (Blackburn *et al.*, 1970; Deslauriers and Smith, 1973), 2′,3′-cyclic mononucleotides (Lavallee and Coulter, 1973; Lapper and Smith, 1973) and some 2′- and 3′-nucleotides (Schleich *et al.*, 1972; Tran-Dinh Son *et al.*, 1972; Tran-Dinh Son and Chachaty, 1973) the unequivocal distinction between gt and tg conformer populations was not made. According to Remin and Shugar (1972), an assignment of the C(5′) methylene protons can be made from chemical shift differences between a nucleoside and the corresponding 3′-nucleotide and results in a preference for gt compared to tg (Table IV).

We have analyzed $J_{4'5'}$ and $J_{4'5''}$ couplings of 2′- and 3′-nucleotides by the same method utilizing equations for calculating conformer populations derived previously (Table VII, Davies and Danyluk, 1974). Since vicinal coupling data are not available for pure rotamers, estimates of J_g = 2.04 Hz and J_t = 11.72 Hz were made from the appropriate Karplus relation (Blackburn *et al.*, 1970). Using the coupling constant data in Table II rotamer populations were calculated for all of the 2′(3′)-nucleotides and are summarized in Table IV. Within the limitations of this analysis, a

[4] Although the results in Table III suggest that the pseudorotational angle is dependent on the base ring in 2′(3′)-ribonucleotides it is clear from the assumptions made for the pseudorotational analysis of furanose rings that part of the difference results from the use of a composite Karplus relation for both purine and pyrimidine derivatives. It can be seen from Table II that for the purine 2′- and 3′-ribonucleotides $\bar{J}_{2'3'}$ = 5.2 (±0.2) Hz and ($\bar{J}_{1'2'} + \bar{J}_{3'4'}$) = 9.1 (±0.2) Hz whereas for the pyrimidine derivatives $\bar{J}_{2'3'}$ = 5.2 (±0.2) Hz and ($\bar{J}_{1'2'} + \bar{J}_{3'4'}$) = 9.8 (±0.1) Hz. Each set of values gives rise to different constants for the Karplus relation (*i.e.*, purines A = 9.9, B = −0.6; pyrimidines A = 10.4, B = −0.9). The results of the pseudorotational analysis on each set of molecules using the appropriate Karplus relation yield values of P and τ for purine and pyrimidine derivatives that are closer together than those listed in Table III.

[5] One Karplus equation was used in the analysis of both sets of data. A change in the expression might be expected because of electronegativity differences for C and N atoms, but this cannot be quantified.

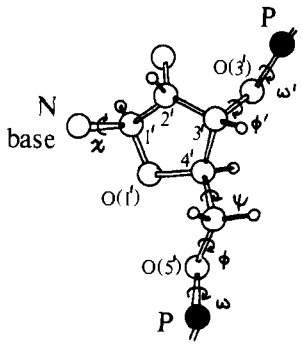


FIGURE 5: Sugar phosphate backbone of nucleic acids. The nomenclature for each torsional angle is that used by Sundaralingam (1969, 1973).

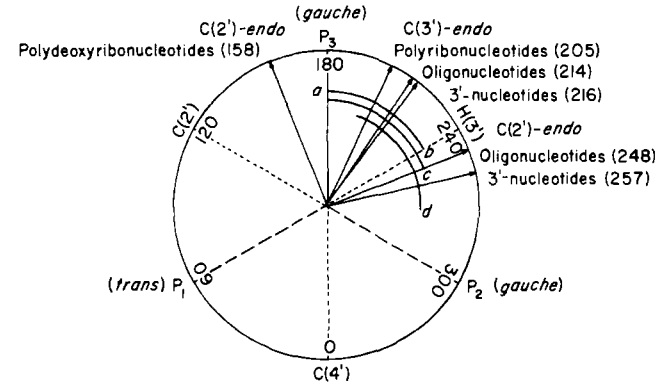


FIGURE 6: Conformational properties of C(3')-O(3') bonds. The diagram is based on definitions of ϕ' angles (Sundaralingam, 1969). In order to preserve consistency with rotamer calculations (Smith *et al.*, 1973), P_1, P_2, and P_3 represent the classical staggered rotamers placing the P-O(3') bond trans, gauche, and gauche with respect to the C(3')-H(3') bond. a, b, c, and d represent results of theoretical calculations: a,b(ϕ', 180–240) represents the stable zone from MO(PCILO) calculation with a global minimum at a($\phi' \sim 180°$) (Pullman *et al.*, 1972; Perahia *et al.*, 1973); c and d represent allowed ϕ' ranges from hard sphere and classical energy calculations for deoxyribonucleotides (c, 180–250°) and ribonucleotides (d, 210–270)° (Lakshiminarayanan and Sasisekharan, 1969; Sasisekharan, 1973).

marked preference is evident for a gg rotamer about C(4')-C(5'). A preferred gg conformer is a feature that recurs in aqueous solution of various nucleosides (Remin and Shugar, 1972), nucleoside 2',3'-cyclic monophosphates (Lapper and Smith, 1973), some pyrimidine 5'-ribonucleotides (Hruska *et al.*, 1973; Wood *et al.*, 1973) and all of the 5'-ribo- and deoxyribonucleotides (Davies and Danyluk, 1974) as well as in the majority of nucleosides and nucleotides in the solid state (Sundaralingam, 1969, 1973). Although temperature change, ionic strength, and solvent nature exert some perturbation on rotamer populations, the changes are relatively small, the gg rotamer varying by less than 10% over a 70° range.

3. Exocyclic Phosphate Group Conformations. A complete conformational description of the sugar–phosphate backbone requires knowledge about rotational properties of five distinct bonding arrangements (Figure 5). Information for three of these angles, ϕ, ψ, ϕ', can be derived from proton magnetic resonance spectroscopy. For example, rotational properties of C(4')-C(5') and C(5')-O(5') were obtained from an analysis of H(4')-H(5'), H(4')-H(5'') and ^{31}P-H(5'), ^{31}P-H(5'') coupling constants (Davies and Danyluk, 1972, 1974; Hruska *et al.*, 1973b). A similar approach is feasible for rotation about C(3')-O(3') and C(2')-O(2'), but the procedures are not as straightforward because the assumption of a threefold potential barrier about these bonds is questionable. Before proceeding with the rotamer analysis it is useful to review relevant crystallographic data for 3'-nucleotides, dinucleoside phosphates, and polynucleotides, and results of theoretical calculations.

The five crystal structures of 3'-nucleotides show that conformations about the C(3')-O(3') bond are restricted to a range of ϕ' from 195 to 269° with a further subdivision into C(2')-endo, $\phi' \sim 257 \pm 8°$ and C(3')-endo, $\phi' \sim 216$ (± 21)°, ranges (Sundaralingam, 1973). Oligonucleotides[6] appear to follow the same pattern with C(3')-endo conformations having $\phi' \sim 214$ (±7)° and C(2')-endo conformations $\phi' \sim 248$ (±4)°. Polynucleotides in the fibrous state exhibit a range of ϕ' 147–223° (Sundaralingam, 1973), with angles again related to furanose ring conformations, *i.e.*, polyribonucleotides, C(3')-endo, $\phi' \sim 205$ (±8)°, and polydeoxyribonucleotides, C(2')-endo, $\phi' \sim 158$ (±12)° or $\phi' = 176$ (±23)°, including the results of C-DNA, 9.3°. Generally the trend is ϕ' (polynucleotides) < ϕ' (3'-nucleotides) which is a consequence of constraints imposed on the former by the helical structure (Sundaralingam, 1969). Such a constraint also explains the reversal in relative values of ϕ' for C(2')-endo and C(3')-endo conformations in going from 3'-nucleotides to polynucleotides. These results are represented in a circular diagram in Figure 6. Figure 6 shows that for polynucleotides as a whole $\phi' \sim 180°$ which places the P-O(3') bond antiperiplanar to the C(3')-C(4') bond and gauche with respect to the C(3')-H(3') bond. On the other hand, the two ϕ' ranges for the 3'-nucleotides lie ±20° on either side of an eclipsed conformation with the P-O(3') bond cis planar to the C(3')-H(3') bond. A conformation corresponding to a classical gauche rotamer only occurs in polynucleotides. Molecular orbital calculations (PCILO) of nucleotide conformations (Pullman *et al.*, 1972; Perahia *et al.*, 1973) have shown that a stable zone exists in a ϕ' range, 180–240°, with a global minimum at $\phi' \sim 180°$, while results using the Extended Hückel theory yield a minimum energy conformation at $\phi' \sim 240°$ (Saran and Govil, 1971). Hard sphere and classical energy calculations do not show trans or gauche as the lowest energy conformation, but instead indicate a range of allowed ϕ' angles depending upon the nature of the furanose ring and on ring pucker (Lakshiminarayanan and Sasisekharan, 1969; Sasisekharan, 1973); the maximum allowed range for ribose derivatives, ϕ' (210–270°), differs from that for deoxyribose derivatives, ϕ' (180–250°). Thus, the theoretical studies indicate energy minima in the same quadrant of Figure 6 with the expected preferred conformation having $\phi' \sim 180$–240°, though significant probability of observing conformations with ϕ' as large as 270° has been suggested (Sasisekharan, 1973). The results of theoretical calculations are, therefore, in broad agreement with X-ray structures though neither method shows conformations in the range of trans or gauche (300) ϕ' values.

An nmr rotamer analysis about C(3')-O(3') has been reported recently for a number of nucleotides using vicinal ^{13}C–^{31}P coupling constants (Smith *et al.*, 1973) as input

[6] Torsional angles (ϕ') are as follows: C(3')-endo conformations; UpA, molecule 1, U(223), molecule 2, U(202), Rubin *et al.*, 1972; GpC, G (209), Day *et al.*, 1973; ApU, molecule 1, A(213), molecule 2, A(220), Rosenburg *et al.*, 1973; ApApA, A(223), A(207), Suck *et al.*, 1973; C(2')-endo conformations; $A_{2'}$ $p_{5'}U$, A(244) Shefter *et al.*, 1969; pTpT, T(252), Camerman *et al.* (1973).

Table V: Fractional Populations of C(3′)-O(3′) Rotamers Calculated from $^3J_{^{31}P,^{13}C}$ and Comparison of Predicted $^3J_{31}$ with Observed Values.

Compd	$^3J_{^{31}P,^{13}C}$ 2′(1′)	$^3J_{^{31}P,^{13}C}$ 4′(3′)	Rotamer Population[a] P_1	P_2	P_3	Calcd[b] $^3J_{^{31}P,H(3')}$	Obsd $^3J_{^{31}P,H(3')}$
UMP-3′	2.5	6.0	0.31	0.04	0.65	7.7	8.0,[c] 8.0,[d] 7.7[e]
UMP-2′	(9)	(3)		Does not fit		Small	6.8[c]
AMP-3′	2.8	4.5	0.53	0.09	0.38	11.9	8.0,[c] 7.4[f]
Poly(U)	5.0	3.0	0.41	0.47	0.12	9.6	8.2[d]

[a] Calculations made using $^3J_t(^{31}P,^{13}C) = 8.0$ Hz and $^3J_g(^{31}P,^{13}C) = 2.3$ Hz (used by Smith *et al.* (1973) for UMP-3′) and equations: $^3J_{^{31}P,C(2')} = P_1J_g + P_2J_t + P_3J_g$, $^3J_{^{31}P,C(4')} = P_1J_g + P_2J_g + P_3J_t$, and $1 = P_1 + P_2 + P_3$. [b] Calculations made using $^3J_t(^{31}P,H) = 20.9$ Hz and $^3J_g(^{31}P,H) = 1.8$ Hz (Blackburn *et al.*, 1973). Calculated $^3J_{^{31}P,H(3')}$ values using $^3J_t(^{31}P,H) = 25$ Hz and $^3J_g(^{31}P,H) = 3$ Hz (Smith *et al.*, 1973) are UMP-3′ (9.8 Hz), AMP-3′ (14.7 Hz), and poly(U) (12.0 Hz). [c] This work. [d] Kreishman and Chan (1971). [e] Schleich *et al.* (1972). [f] Tran-Dinh Son and Chachaty (1973).

data. Assuming rapid rotation among the three staggered conformers, designated P_1, P_2, P_3 in Figure 6, and that vicinal $J_{^{13}C,^{31}P}$ couplings for each pure conformer are identical with those for rigid 3′,5′-cyclic nucleotides, *i.e.*, $J_g = 2.3$ Hz and $J_t = 8.0$ Hz, Smith and coworkers (1973) calculated rotamer populations for UMP-3′. These are summarized in Table V along with our calculation for AMP-3′ and poly(U). The results show a small population of P_2 rotamer, (P-O(3′) bond antiperiplanar to the C(3′)-C(2′) bond, ϕ' ~300), but significant populations for P_1(ϕ' ~60) and P_3 (ϕ' ~180) rotamers in UMP-3′ and AMP-3′; poly(U), in contrast, has approximately equal populations for P_1 and P_2 and a much smaller population of P_3. Thus, on an nmr time scale there is considerable rotational freedom about C(3′)-O(3′) in 3′-nucleotides. However, an evident discrepancy exists between rotamer populations deduced from nmr data and conformational results from theoretical calculations and X-ray diffraction measurements (Figure 6). Perhaps most surprising is the significant populations determined for the P_1 conformer (Table V), whose existence is not indicated by the other approaches.

A check of the ^{13}C results can be made by calculating the vicinal ^{31}P-H(3′) coupling constants utilizing populations from Table V, and $J_t = 20.9$ Hz, $J_g = 1.8$ Hz obtained from 3′,5′-cyclic nucleotides (Blackburn *et al.*, 1973). For rapid rotation among P_1, P_2, and P_3, $J_{^{31}P,H(3')}$ is given by

$$J_{^{31}P,H(3')obsd} = P_1J_t + P_2J_g + P_3J_g \qquad (5)$$

Predicted and observed values for this coupling are given in the last two columns in Table V. The calculated magnitudes for $J_{^{31}P,H(3')}$ will depend to some extent on values selected for J_t and J_g in H-C-O-P and C-C-O-P fragments, but within this restriction the agreement between calculated and observed couplings is satisfactory for UMP-3′ only.

Several alternative rotamer possibilities consistent with $J_{obsd} \sim 8.0$ Hz might also be considered. These include fixed conformers with ϕ' values of 0 and 120° (dihedral angle ~120-125°) and 210 and 270° (dihedral angles ~30-35°) assuming a Karplus relation for coupling in the H-C-O-P fragment (Blackburn *et al.*, 1973). The first two possibilities can be eliminated because they involve eclipsed P-O and C-C bonds while the latter possibilities are inconsistent with chemical shift (*cf.* below) and long-range coupling constant data. Finally, free rotation among the three staggered rotamers, Figure 6, will produce a coupling of ~8.0 Hz for ^{31}P-H(3′), very close to observed values.

A further insight into rotamer properties about C(3′)-O(3′) and C(2′)-O(2′) can be gained from chemical shift considerations for relevant ring protons in 2′(3′) nucleotides and related nucleosides and deoxynucleotides. Pertinent data are summarized in Table VI. As expected, shifts for the nucleotides are at lower field than in nucleosides, with the greatest deshielding found for H(3′), H(2′) in 3′- and 2′-nucleotides, respectively. For all of the 3′-nucleotides the deshielding of H(4′) (0.1-0.17 ppm) is almost twice that for H(2′) (0.055-0.071 ppm) and is due to a closer juxtaposition of H(4′) to the phosphate group in two staggered conformations (P_1, P_3, Figure 6); a like approach of the phosphate group to H(2′) occurs in only one staggered conformer (P_2). Unfortunately, these shift differences are not suitable for quantitative evaluation of rotamer populations, but they strongly suggest a uniform population of each rotamer, and/or free rotation. This possibility is further supported by an examination of chemical shift differences for the deoxyribose ring in 3′-deoxynucleotides and deoxynucleosides (Table VI). Shifts of particular interest are those for H(4′) and H(2″) (Figure 1). Dreiding structural models show that H(4′) and H(2″) of the deoxyribose ring are symmetrically placed with respect to the C(3′)-O(3′) bond. Since it is expected that the furanose ring conformations and syn-anti ratios do not differ greatly between 3′-deoxyribonucleotides and the corresponding deoxyribonucleosides, the shifts for H(4′) and H(2″) can serve as monitors of phosphate group orientation. The results in Table VI show that in fact H(4′) and H(2″) exhibit approximately equal downfield shifts and that H(2′) is deshielded by nearly the same amount in 3′-deoxyribonucleotides and ribonucleotides. The combined chemical shift results for 3′-ribo- and deoxyribonucleotides thus strongly point to a rotamer equilibrium between three equally (or nearly equally) populated rotamers, *i.e.*, P_1, P_2, P_3. Alternate possibilities such as preferred P_1 or preferred P_2 and P_3 (equally populated) are not supported by $^3J_{^{31}P,H(3')}$ and $^3J_{^{31}P,^{13}C}$ data, and H(2′)-H(4′) shift differences, respectively. Finally, rapid rotation between equally populated rotamers would account for the absence of any significant temperature dependence for $J_{^{31}P,H(3')}$ (Schleich *et al.*, 1972), and present results.

The nmr data thus show that the C(3′)-O(3′) bond of 3′-nucleotides has a much greater degree of rotational flexibility than found for C(4′)-C(5′) and C(5′)-O(5′) bonds of 5′-nucleotides (Davies and Danyluk, 1974; Sarma *et al.*, 1973; Wood *et al.*, 1973), a result which has important

Table VI: Chemical Shift Differences between 2′(3′)-Nucleotides and Nucleosides in Aqueous Solution.[a]

Compounds	Furanose Ring Protons							Base Rings	
	1′	2′	2″	3′	4′	5′	5″	H-6(H-8)	H-5(H-2)
UMP-2′[b] − U[c]	0.067	0.328		0.129	−0.001	−0.046	−0.010	−0.046	0.007
CMP-2′[b] − C[c]	0.055	0.316		0.107	−0.026	−0.078	0.038	−0.081	−0.023
AMP-2′[b] − A[d]	0.091	0.286[e]		0.153	0.008	−0.002	0.006	0.040	−0.042
GMP-2′[b] − G[d]	0.085	0.340[e]		0.16_6	0.02_1	-0.04_2	0.01_6	−0.016	
UMP-3′[b] − U[c]	0.026	0.055		0.263	0.100	−0.014	0.049	0.004	0.007
CMP-3′[b] − C[c]	0.035	0.071		0.288	0.093	−0.012	0.055	0.017	0.008
AMP-3′[b] − A[d]	0.059	0.077[e]		0.311	0.172	0.019	0.100	0.051	−0.008
GMP-3′[b] − G[d]	0.011	0.039[e]		0.33_9	0.16_5	0.00_6	0.10_8	0.009	
UMP-3′[f] − U(23°)[f]	0.026	0.060		0.275	0.101	−0.009	0.049	−0.017	0.013
UMP-3′(88°)[f] − U(80°)[f]	0.005	0.063		0.219	0.107	0.015	0.057	−0.033	−0.015
β-ψUMP-3′(23°)[f] − βψU(30°)[f]	0.041	0.062		0.292	0.107	0.010	0.059	0.034	
β-ψUMP-3′(75°)[f] − βψU(70°)[f]	0.046	0.074		0.327	0.118	0.018	0.066	0.048	
dAMP-3′[d] − dA[d]	0.057	0.033	0.162	0.277	0.188	0.005	0.065	−0.002	−0.062
dGMP-3′[d] − dG[d]	0.008	0.078	0.193	0.307	0.158	0.006	0.009	0.008	
dCMP-3′[d] − dC[d]	0.014	0.050	0.149	0.270	0.143	0.027	0.041	0.010	0.019
TMP-3′[d] − T[d]	−0.007	0.038	0.170	0.303	0.151	0.005	0.017	0.016	0.002

[a] Downfield shifts have positive sign. [b] This work. [c] Hruska *et al.* (1973a). [d] Davies and Danyluk, unpublished results. [e] Approximate value due to estimation of H(2′) signal of adenosine and guanosine which is masked by HDO signal (~4.75 ppm). [f] Schleich *et al.* (1972).

structural implications for higher chain-length nucleic acid derivatives.

A rotamer analysis about C(2′)-O(2′) can be made from $J_{^{31}P,H(2')}$ magnitudes. The observed couplings of 6.8–7.0 Hz indicate a slight preference for the gauche compared to the trans conformer. As noted for 3′-nucleotides, a single coupling constant cannot be used to differentiate relative populations of the two gauche conformers. ^{13}C nmr studies on UMP-2′ (Smith *et al.*, 1973) report values of 9 and 3 Hz for ^{31}P-C(1′) and ^{31}P-C(3′) couplings, respectively. These values are best interpreted if the C(3′)-gauche, C(1′)-trans conformer comprises a major fraction of the rotamer population in UMP-2′. In this conformation P(2′)-O(2′) is gauche to C(2′)-H(2′) and is consistent with $^3J_{^{31}P,H(2')}$ = 6.8 Hz, eq 5. The similarity in ^{31}P-H(2′) couplings in the series of 2′-nucleotides supports a C(1′) trans conformation in all of the molecules. Further substantiating evidence is provided by chemical shift differences between pyrimidine 2′-nucleotides and nucleosides (Table VI). A greater downfield shift is exerted by the phosphate group on H(3′)(0.107–0.129 ppm) compared to H(1′) (0.055–0.067 ppm), a result which follows directly for a preferred C(3′)-gauche, C(1′)-trans conformation about C(2′)-O(2′). Analogous behavior is found for purine 2′-ribonucleotides which suggests that the same conformation about the C(2′)-O(2′) bond, (C(3′)g, C(1′)t), is found for all 2′-ribonucleotides in solution.

4. Long-Range ^{31}P–H Couplings. The magnitudes of long-range homo- and heteronuclear couplings often provide useful information about the stereospecific arrangement of bonds along the coupling path. Thus, Hall and coworkers (Hall and Malcolm, 1972a,b; Hall and Donaldson, 1972) reported observable ^4J couplings in H-C-C-O-P fragments with an all-trans, planar (W) coupling path. Long-range four-bond ^{31}P-H couplings have been found in several nucleotides with a coupling path along H(4′)-C(4′)-C(5′)-O(5′)-P (Hruska *et al.*, 1973; Wood *et al.*, 1973; Sarma *et al.*, 1973). Furthermore, the latter workers showed that, as the gg conformer about C(4′)-C(5′) and C(5′)-O(5′) bonds decreased $^4J_{^{31}P,H(4')}$ decreased also. A similar trend was observed for all common 5′-ribo- and deoxyribonucleotides (Davies and Danyluk, 1974) along with a correlation between the magnitude of $^4J_{^{31}P,H(4')}$ and the S conformer preference of the ribose ring.

For 2′- and 3′-ribonucleotides four-bond phosphorus proton couplings are possible with H(1′), H(3′), and H(2′), H(4′), respectively. Their magnitudes will depend upon rotamer properties about C(2′)-O(2′) and C(3′)-O(3′), and, to a lesser extent, upon ribose ring conformation. Such couplings have not been measured previously for 2′- and 3′-ribonucleotides though earlier workers have reported four-bond ^{31}P-H J values in some cyclic 2′,3′-ribonucleotides ($^4J_{^{31}P,H(1')}$, Davies and Danyluk, 1972; Lavallee and Coulter, 1973) and in thymidine 3′,5′-cyclic monophosphate (Blackburn *et al.*, 1973). For TMP-3′,5′ four-bond couplings to ^{31}P have values of H(2′) (0.5 Hz), H(2″) (0.5 Hz), and H(4′) (<0.2 Hz) and a five-bond coupling of 0.5 Hz to H(1′). The TMP-3′,5′ ribose ring approximates to a C(3′)-endo, C(4′)-exo twist conformation which provides an approximate all-trans coupling path from ^{31}P to H(1′) but only part of a planar path to both H(2′) and H(2″). Thus $^4J_{^{31}P,H}$ is smaller in cyclic nucleotides than in model compounds with complete planar W conformations for H-C-C-O-P bond systems (2.4–2.7 Hz, Hall and Malcolm, 1972a,b).

Qualitatively, a coupling of $^4J_{^{31}P,H(2')}$ = 1.0 Hz for AMP-3′ indicates a substantial fraction of the total conformer populations along the flexible P(3′)-O(3′)-C(3′)-C(2′)-H(2′) bonding system exists in a planar W form. Dreiding molecular models show that such a coupling path is particularly favored for a ribose ring in the S conformation (C(2′)-endo) and a P(3′)-O(3′) bond antiperiplanar to the C(3′)-C(2′) bond (conformer P_2, Figure 6). Our data for AMP-3′ (70°) show a preferred S ribose conformation (66:34, Table III), whereas pyrimidine 3′-nucleotides, which show no case of long-range $^4J_{^{31}P,H(2')}$ couplings, favor

an N conformation (56:44, Table III). In line with this trend $^4J_{^{31}P,H(2')}$ for GMP-3′ is less than 0.5 Hz and shows a less preferred S ribose conformation than AMP-3′. The small value of $^4J_{^{31}P,H(4')} = 0.5$ Hz for AMP-3′ also suggests a substantial contribution from a planar W conformation along the flexible H(4′)-C(4′)-C(3′)-O(3′)-P(3′) bonding system. Dreiding molecular models show that a complete planar W coupling path is not possible, but the closest approximation occurs for a furanose ring in either the N or S conformer with the P(3′-O(3′) bond antiperiplanar to the C(3′)-C(4′) bond (conformer P_3, Figure 6). Similar four-bond long-range couplings (0.7–0.9 Hz) have been observed in 2-oxo-1,3,2-dioxaphosphorinane derivatives (Hall and Malcolm, 1972a,b) in which only three bonds exhibit an all-trans planar conformation. The composite picture which these results point to is of an exocyclic 3′-phosphate group which exists in both P_2 and P_3 conformations to an appreciable extent. This conclusion agrees with results of rotamer calculations about C(3′)-O(3′) (*loc cit*).

For 2′-nucleotides the absence of a detectable $^4J_{^{31}P,H}$ coupling with protons at C(1′) and C(3′) positions is in accord with the existence of a preferred C(3′)-endo, C(1′)-trans conformation along the C(3′)-C(2′)-O(2′)-C(1′) fragment (preceding section). In this conformation no favorable coupling path exists for ^{31}P-H(3′) or ^{31}P-H(1′) couplings and hence the magnitudes of such couplings will be very small in 2′-nucleotides.

5. Conformation about the Glycosidic Bond. An extensive accumulation of crystallographic data for nucleosides and nucleotides shows that the torsion angle, χ_{CN}, between base and ribose rings falls into two relatively narrow ranges, designated as syn and anti conformations (Donohue and Trueblood, 1960; Haschemeyer and Rich, 1967; Sundaralingam, 1969). For β anomers a further correlation exists between χ_{CN} and the furanose ring conformation (Haschemeyer and Rich, 1967; Altona and Sundaralingam, 1972; Sundaralingam 1969, 1973). In the few published crystal structures for 3′-nucleotides the anti conformation is preferred, and a correlation is again noted between χ_{CN} and the furanose ring conformation, *i.e.*, χ_{CN} (C(2′)-endo) = 42 (±3)° and χ_{CN} (C(3′)-endo) = 16 (±12)°, (Sundaralingam, 1973). Recent X-ray crystallographic data[7] of dinucleoside phosphates, a dinucleotide, and a trinucleoside diphosphate exhibit a similar trend with χ_{CN} (C(2′)-endo) = 39 (±11)° and χ_{CN} (C(3′)-endo) = 20 (±11)°.

Analogous quantitative information for 2′- and 3′-ribonucleotides is not yet available in solution although recent nuclear Overhauser measurements are in accord with a higher syn population (~70:30) for AMP-2′, AMP-3′ and GMP-2′, GMP-3′, and a preferred anti conformation for AMP-5′ (70:30) and GMP-5′ (53:47) (Tran-Dinh Son *et al.*, 1972; Tran-Dinh Son and Chachaty, 1973; Guéron, 1973). Differences in the conformational equilibrium will be reflected in chemical shifts of the ribose ring protons.

A recent pmr study of cyclic AMP-3′,5′ and its eight-substituted derivatives and both cyclic GMP-3′,5′ and cyclic IMP-3′,5′ and their 8-bromo derivatives (Schweizer and Robins, 1973) has shown that the eight-substituted derivatives prefer a syn conformation whereas the parent cyclic nucleotide prefers an anti conformation. For 8-bromo derivatives in the syn conformation a downfield shift occurs for ribose ring protons, *i.e.*, H(2′) (0.2–0.3 ppm), H(3′) (0.24–0.51 ppm), and H(4′) (0.02–0.08 ppm), relative to shifts in the anti form. In cyclic 3′,5′-mononucleotides the ribose ring is locked in a C(3′)-endo–C(4′)-exo conformation (Blackburn *et al.*, 1973) accounting for the greater downfield effect on H(3′) than H(2′). At present there is no comparable information for purine nucleotides with ribose rings locked in the C(2′)-endo conformation; otherwise, chemical shift effects on ribose ring protons might be used to monitor syn ⇌ anti equilibria of purine nucleotides in solution when account is taken of the N ⇌ S equilibrium of the ribose ring.

In earlier work (Davies and Danyluk, 1974), it was shown that a phosphate group in a 5′ position deshielded base ring protons in purine and pyrimidine nucleotides relative to respective 2′- and 3′-nucleotides, as would be expected for anti conformations in these molecules. A further observation of interest is the deshielding of ribose protons in purine 5′-nucleotides relative to pyrimidine derivatives, the effect being greatest at H(2′) (0.359–0.421 ppm) with smaller changes at H(3′) (0.123–0.165), H(4′) (0.096–0.109), and H(1′) (0.063–0.113). Since the S (C(2′)-endo) conformation is preferred (60:40) in these molecules a greater purine deshielding effect is expected at H(2′) than H(3′) or H(4′).

A similar trend is found for purine and pyrimidine 2′- and 3′-nucleotides as is evident from chemical shift differences compiled in Table VII. The results show that the purine ring deshields the ribose ring protons, in the order H(2′) > H(3′) > H(4′) > H(1′) H(5′) (5″). All of the furanose proton shift differences (purine–pyrimidine) show a greater displacement to lower field in 3′-nucleotides compared to the 5′-nucleotides,[8] *i.e.*, H(1′) (0.071 ppm), H(2′) (0.049 ppm), H(3′) (0.059 ppm), H(4′) (0.081) ppm. The origin of these differences is not known though the trends are consistent with a change in syn ⇌ anti equilibrium of 5′-nucleotides (~40:60) compared to 3′-nucleotides (~70:30) (Tran-Dinh Son *et al.*, 1972; Tran-Dinh Son and Chachaty, 1973; Guéron, 1973), the downfield shifts of purine derivatives being greater in the syn conformation than in the anti conformation (Schweizer and Robins, 1973). The approximately equal effect on H(2′) and H(3′) is a consequence of dynamic equilibrium of the furanose ring, a point which would be of considerable interest when chemical shifts for locked C(2′)-endo nucleotides become available. Similar considerations apply to the chemical shift differences of purine and pyrimidine 2′-nucleotides (Table VII) relative to those for 5′-nucleotides,[8] in line with results from nuclear Overhauser experiments which show that the syn conformation is favored for purine 2′-ribonucleotides (~70:30) compared to corresponding 5′-nucleotides.

6. Summary of Conformational Deductions. The nmr results of this work lead to a number of conformational conclusions for 2′(3′)-ribonucleotides. (i) An analysis of coupling constant data in terms of pseudorotational parameters reveals that the D-ribose rings of purine 2′(3′)-ribonucleot-

[7] Glycosidic torsional angles (χ_{CN}) are as follows: C(3′)-endo conformations: UpA, molecule 1, A(49), U(20), molecule 2, A(36), U(10), Rubin *et al.*, 1972; ApU, molecule 1, A(7), U(29), molecule 2, A(2), U(30), Rosenburg *et al.*, 1973; GpC, G(13), C(25), Day *et al.*, 1973; $A_2'p_5'U$, U(5), Shefter *et al.*, 1969; ApApA, A(7), A(24), A(21), Suck *et al.*, 1973; C(2′)-endo conformations; $A_2'p_5'U$, A(55), Shefter *et al.*, 1969; pTpT, T(27), T(34), Camerman *et al.*, (1973).

[8] The average values of chemical shift differences between purine and pyrimidine 5′-nucleotides (Table II, Davies and Danyluk, 1974) are H(1′) (0.014 ppm), H(2′) (0.397), H(2″) (0.225), H(3′) (0.158), H(4′) (0.093).

Table VII: Chemical Shift Differences between Purine and Pyrimidine 2′- and 3′-Ribonucleoside Monophosphates.[a]

	1′	2′	2″	3′	4′	5′	5″
δ(AMP-2′ − CMP-2′)	0.160	0.405		0.250	0.182	0.049	0.050
δ(AMP-3′ − CMP-3′)	0.148	0.441		0.227	0.229	0.004	0.051
δ(GMP-2′ − UMP-2′)	0.035	0.422		0.220	0.117	0.010	0.011
δ(GMP-3′ − UMP-3′)	0.002	0.395		0.259	0.160	−0.023	0.036
δ(dAMP-3′ − TMP-3′)[b]	0.176	0.446	0.195	0.173	0.227	0.011	0.072
δ(dGMP-3′ − dCMP-3′)[b]	0.013	0.493	0.113	0.223	0.090	−0.19	0.002
Mean[c]	0.085	0.444	0.154	0.216	0.174		

[a] Positive values denote downfield chemical shifts. [b] D. B. Davies, to be published. [c] Average value for 3′-nucleotides.

ides favor an S type conformation [C(2′)endo, C(3′)exo] over an N-type in the approximate ratio 60:40, while the results for pyrimidine 2(3′)-ribonucleotides show a 50:50 equilibrium mixture. In both sets of nucleotides a rapid determination of furanose ring equilibrium compositions can be made directly by evaluating the ratio $J_{1'2'}:J_{3'4'}$. (ii) Values for the pseudorotational parameters in solution generally fall in ranges reported for mononucleotides in the crystalline state, with no significant change of ring pucker or pseudorotational angle occurring in change of state. (iii) Proton couplings across the C(4′)–C(5′) bond were analyzed in terms of a simple gg, tg, gt rotamer conformational model. In all nucleotides, there is a marked preference for the gg rotamer. It was also found that the gt rotamer is more favored than the tg rotamer. Small differences in rotamer populations about the C(4′)–C(5′) bond and in the N $\rightleftharpoons$ S equilibrium of 2′(3′)-nucleotides are not sufficient to reveal an interdependence between furanose ring equilibrium and rotamer populations of the exocyclic group, as noted in 5′-nucleotides. (iv) Proton–phosphorus couplings across the C(2′)–O(2′) and C(3′)–O(3′) bonds were analyzed in terms of a conformational equilibrium between gauche and trans conformers. In 2′-nucleotides a definite preference is found for the C(3′)g, C(1′)t conformer in agreement with available ^{13}C nmr data (Mantsch and Smith, 1972; Smith *et al.*, 1973). For 3′-nucleotides, no clear distinction can be made between the preferred H(3′)g, C(4′)t, and H(3′)t conformations derived from ^{13}C nmr studies (Smith *et al.*, 1973) or the expected preferred H(3′)g, C(4′)t, and H(3′)g, C(2′)t conformations observed in the crystal state (Sundaralingam, 1973) and predicted by theoretical calculations (Pullman *et al.*, 1972; Perahia *et al.*, 1973; Lakshiminarayan and Sasisekharan, 1969; Sasisekharan, 1973). However, the coupling constant data, when combined with chemical shift results for deoxynucleosides and nucleotides, are mostly in agreement with nearly free rotation and approximately equal rotamer population about C(3′)–O(3′). (v) Chemical shift differences of furanose ring protons of purine and pyrimidine 2′(3′)-nucleotides compared to 5′-nucleotides can be interpreted by a more favored syn orientation in 2′(3′)-nucleotides in agreement with previous nuclear Overhauser experiments (Tran-Dinh Son *et al.*, 1972; Tran-Dinh Son and Chachaty, 1973; Guéron, 1973).

In total the nmr results show only slight variations in syn–anti orientation, ribose ring conformation, and in exocyclic C(5′) group orientation in going from 5′-nucleotides to 2′- and 3′-nucleotides. A nucleotidyl unit incorporating these conformational features thus appears to be quite stable dynamically with the gg conformation about C(4′)–C(5′) being particularly favored in all nucleotides and nucleosides. One distinctive difference between 5′-nucleotides and 3′-nucleotides is the greater rotamer flexibility present in the exocyclic 3′-phosphate group of the latter. The existence of such a flexibility has been proposed from X-ray diffraction results (Rubin *et al.*, 1972). Finally the present nmr results provide a consistent data base for nmr analysis of more complex oligonucleotides in solution.

References

Altona, C., and Sundaralingam, M. (1972), *J. Amer. Chem. Soc. 94,* 8205.

Altona, C., and Sundaralingam, M. (1973), *J. Amer. Chem. Soc. 95,* 2333.

Blackburn, B. J., Grey, A. A., Smith, I. C. P., and Hruska, F. E. (1970), *Can. J. Chem. 48,* 2866.

Blackburn, B. J., Lapper, R. D., and Smith, I. C. P. (1973), *J. Amer. Chem. Soc. 95,* 2873.

Camerman, N., Fawcett, J. K., and Camerman, A. (1973), *Science 182,* 1142.

Davies, D. B., and Danyluk, S. S. (1972), International Symposium on NMR Spectroscopy, University of Surrey.

Davies, D. B., and Danyluk, S. S. (1974), *Biochemistry, 13,* 4417.

Day, R. O., Seeman, N. C., Rosenburg, J. M., and Rich, A. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 849.

Deslauriers, R., and Smith, I. C. P. (1973), *Can. J. Chem. 51,* 833.

Donohue, J., and Trueblood, K. N. (1960), *J. Mol. Biol. 2,* 363.

Govil, G., and Saran, A. (1971), *J. Theor. Biol. 30,* 621.

Grey, A. A., Smith, I. C. P., and Hruska, F. E. (1971), *J. Amer. Chem. Soc. 93,* 1765.

Guéron, M. (1973), *Ann. N. Y. Acad. Sci. 222,* 307.

Hall, L. D., and Donaldson, B. (1972), *Can. J. Chem. 50,* 2111.

Hall, L. D., and Malcolm, R. B. (1972a), *Can. J. Chem. 50,* 2092.

Hall, L. D., and Malcolm, R. B. (1972b), *Can. J. Chem. 50,* 2102.

Haschemeyer, A. E. V., and Rich, A. (1967), *J. Mol. Biol. 27,* 369.

Hruska, F. E., Grey, A. A., and Smith, I. C. P. (1970), *J. Amer. Chem. Soc. 92,* 4088.

Hruska, F. E., Mak, A., Singh, H., and Shugar, D. (1973a), *Can. J. Chem. 51,* 1099.

Hruska, F. E., Wood, D. J., Mynott, R. J., and Sarma, R. H. (1973b), *Fed. Eur. Biochem. Soc. 31,* 153.

Jardetzky, C. D. (1962), *J. Amer. Chem. Soc. 84,* 62.
Kreishman, G. P., and Chan, S. (1971), *Biopolymers 10,* 159.
Lakshiminarayanan, A. V., and Sasisekharan, V. (1969), *Biopolymers 8,* 489.
Lapper, R. D., and Smith, I. C. P. (1973), *J. Amer. Chem. Soc. 95,* 2880.
Lavallee, D. K., and Coulter, C. L. (1973), *J. Amer. Chem. Soc. 95,* 576.
Mantsch, H. H., and Smith, I. C. P. (1972), *Biochem. Biophys. Res. Commun. 46,* 808.
Perahia, D., Saran, A., and Pullman, B. (1973), in Conformation of Biological Molecules and Polymers, Proceedings of the Vth Jerusalem Symposium, Bergmann, E. D., and Pullman, B., Ed., New York, N.Y., Academic Press, p 225.
Pullman, B., Perahia, D., and Saran, A. (1972), *Biochim. Biophys. Acta 269,* 1.
Remin, M., and Shugar, D. (1972), *Biochem. Biophys. Res. Commun. 48,* 636.
Rosenburg, J. M., Seeman, N. C., Kim, J. J. P., Suddath, F. L., Nicholas, H. B., and Rich, A. (1973), *Nature (London) 243,* 150.
Rubin, J., Brennan, T., and Sundaralingam, M. (1972), *Biochemistry 11,* 3112.
Saran, A., and Govil, G. (1971), *J. Theor. Biol. 33,* 407.
Sarma, R. H., Mynott, R. J., Wood, D. J., and Hruska, F. E. (1973), *J. Amer. Chem. Soc. 95,* 6457.
Sasisekharan, V. (1973), *in* Conformation of Biological Molecules and Polymers, Proceedings of the Vth Jerusalem Symposium, Bergmann, E. D., and Pullman, B., Ed., New York, N.Y., Academic Press, p 247.
Schleich, T., Blackburn, B. J., Lapper, R. D., and Smith, I. C. P. (1972), *Biochemistry 11,* 137.
Schweizer, M. P., and Robins, R. K. (1973), *in* Conformations of Biological Molecules and Polymers, Proceedings of the Vth Jerusalem Symposium, Bergmann, E. D., and Pullman, B., Ed., New York, N.Y., Academic Press, p 329.
Shefter, E., Barlow, M., Sparks, R. A., and Trueblood, K. N. (1969), *Acta Crystallogr., Sect. B 25,* 895.
Smith, I. C. P., Mantsch, H. H., Lapper, R. D., Deslauriers, R., and Schleich, T. (1973), *in* Conformations of Biological Molecules and Polymers, Proceedings of the Vth Jerusalem Symposium, Bergmann, E. D., and Pullman, B., Ed., New York, N.Y., Academic Press, p 381.
Suck, D., Manor, P. C., Germain, G., Schwalbe, C. H., Weimann, G., and Saenger, W. (1973), *Nature (London), New Biol. 246,* 161.
Sundaralingam, M. (1969), *Biopolymers 7,* 821.
Sundaralingam, M. (1973), *in* Conformations of Biological Molecules and Polymers, Proceedings of the Vth Jerusalem Symposium, Bergmann, E. D., and Pullman, B., Ed., New York, N.Y., Academic Press, p 381.
Tran-Dinh Son, and Chachaty, C. (1973), *Biochim. Biophys. Acta 335,* 1.
Tran-Dinh Son, Guschlbauer, W., and Guéron, M. (1972), *J. Amer. Chem. Soc. 94,* 7903.
Tran-Dinh Son, Thiery, J., Guschlbauer, W., and Dunand, J.-J. (1972), *Biochim. Biophys. Acta 281,* 289.
Wood, D. J., Hruska, F. E., Mynott, R. J., and Sarma, R. H. (1973), *Can. J. Chem. 51,* 2571.
Wood, D. J., Mynott, R. J., Hruska, F. E., and Sarma, R. H. (1973), *Fed. Eur. Biochem. Soc. 34,* 323.

Hydrogen Bonding Abilities of 2,4-Dithiouridine Derivatives†

J. Pitha* and K. H. Scheit

ABSTRACT: The base pairing ability of a di-2,4-thiouridine derivative was studied in carbon tetrachloride solutions by the methods of infrared spectroscopy. The strength of the association by hydrogen bonding was found to decrease in the following order: adenine–uracil, adenine–di-2,4-thiouracil, uracil–uracil, and di-2,4-thiouracil–di-2,4,-thiouracil. These findings contrast with the previously demonstrated fact that poly(s^2s^4U) is strongly self-associated and does not form a complex with poly(A). To correlate these results, it is proposed that long range stabilizing forces are acting between the di-2,4-thiouracil residues in polynucleotide chains. This assumption also explains the existence of an ordered structure in the alternating copolymer poly(s^2s^4U-A).

The stability of polynucleotide structures and their complexes depends on a number of different molecular interactions. The importance of hydrogen bonding was recognized earliest; the vertical interaction between bases, the restricted rotation around the chemical bonds in the polymer backbone, and ion–ion interactions were fully recognized and studied later (Felsenfeld and Miles, 1967; Inners and Felsenfeld, 1970; Pullman and Pullman, 1969; Eichhorn, 1973, and references therein). Recently one of us reported an interesting change in the properties of poly(U) occurring when the oxygens of uracil are substituted by sulfur atoms. Poly(s^2s^4U),[1] unlike poly(U) or poly(s^4U), forms a very sta-

† From the Laboratory of Molecular Aging, National Institutes of Health, National Institute of Child Health and Human Development, Gerontology Research Center, Baltimore City Hospitals, Baltimore, Maryland 21224, and Max-Planck-Institut für Biophysikalische Chemie, Karl-Friedrich-Bonhoeffer-Institut, Abteilung Molekulare Biologie, Göttingen. *Received March 25, 1974.*

[1] Abbreviations used are: $Ac_3s^2s^4U$, tri-2′,3′,5′-*O*-acetyl-2,4-dithiouridine; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid). Position of thio substitution is noted by a numerical index; ν(XH), stretching vibration of X–H bond.