A Nuclear Magnetic Resonance Study of the Molecular Conformation of β-Pseudouridine in Aqueous Solution

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Abstract: A computer analysis of the entire 100-MHz nmr spectrum of the modified nucleoside β-pseudouridine, β-ψ, in aqueous solution is reported. The analysis was confirmed by spin-tickling and spin-decoupling experiments. The results are used to determine a model for the conformation of the nucleoside. An equilibrium between various puckered ring conformations is proposed. The populations of the various rotamers about the $C_{4'}$–$C_{5'}$ bond are evaluated; a preference for the *gauche–gauche* rotamer is indicated. Comparison with the available data for uridine indicates that the two compounds have almost identical ribose conformations, and that the uracil moiety in β-ψ is present as the *anti* conformer. Lack of variation in either the chemical shifts or spin–spin coupling constants of β-ψ over the temperature range 30–70° demonstrates that no conformation changes occur. The effect of added purine on the nmr parameters of β-ψ shows that base-stacked complexes are formed, and that the complexes are very similar to those formed between uridine and purine.

Nuclear magnetic resonance spectroscopy (nmr) has been successful in providing information about the structures of nucleic acids and their components.[2-19] The most fruitful studies have been those on small molecules whose nmr lines are well resolved. To date attention has been given predominantly to the major components of DNA and RNA (uracil, thymine, guanine, cytosine, adenine, and their nucleosides and nucleotides). Spectroscopic research on the minor constituents of tRNA such as pseudouridine, 2′-O-methylcytidine, and N^6-(Δ^2-isopentenyl)adenosine has been limited.[10,20] Pseudouridine occurs in all the tRNA species sequenced to date.[21,22] It is always present in the sequence TψC in the right-hand loop of the cloverleaf model of the structure;[23,24] in some species of tRNA, such as $tRNA^{Tyr}$ from yeast, it occurs in the anticodon region and recognizes adenosine in a trinucleotide message.[24] It is a unique component of tRNA in that it is a C-glycoside, whereas all other nucleosides occur naturally as N-glycosides. The natural anomer, β-ψ, has the structural formula shown in Figure 1; also shown is the α anomer, α-ψ (5-α-D-ribofuranosyluracil)[25] and its N-glycosidic isomer uridine, U (1-β-D-ribofuranosyluracil). Evidence for the chemical structures of the anomeric species, in particular for the point of attachment of the uracil to the ribose, has been reviewed.[26,27] An early nmr study verified the C–C attachment.[28]

We describe here a complete analysis of the 100-MHz spectrum of β-ψ and the resultant model for its molecular conformation.[29] Spectral analysis was confirmed by computer simulation and double-irradiation experiments. A complete analysis yields all the chemical shifts and proton coupling constants, and reveals subtle details about the three-dimensional arrangement of the atoms in the molecule. In particular, a long-range spin–spin coupling interaction between H_6 and $H_{1'}$ is found, adding further evidence for the structures given in Figure 1. The magnitude of this interaction is discussed with regard to the relative orientations of the ribose and uracil moieties as determined by rotation about the glycoside bond. Consideration of the spin–spin couplings between the ribose protons leads to a three-dimensional model for the furanose ring. The lack of a significant temperature dependence for either coupling constants or chemical

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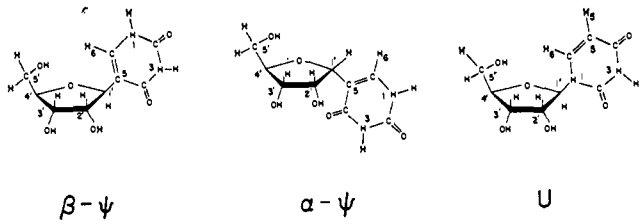

Figure 1. Structural formulas of β-pseudouridine, α-pseudouridine, and uridine.

shifts suggests that no drastic conformational changes take place over the range 30–70°. In addition, nmr spectra obtained in the presence of purine demonstrate the formation of base-stacked complexes similar to those found for uridine.[30]

Experimental Section

β-ψ and α-ψ (100% anomeric purity) and purine were obtained from Calbiochem and used without further purification. The anomeric purity was confirmed by consideration of the nmr data (*vide infra*). The internal reference, 3-trimethylsilylpropanesulfonic acid, sodium salt (DSS), was a product of E. Merck, Germany. The nucleosides were 0.12 *M* in D_2O containing 0.15 *M* DSS. Spectra were run at different concentrations of β-ψ to ensure that the data were concentration independent. The pD was adjusted with a Beckman pH meter, by addition of small amounts of dilute NaOD and DCl (pD = pH + 0.40).[31] The samples were lyophilized three times with D_2O to reduce the concentration of HDO whose resonance might obscure peaks of interest. Proton magnetic resonance spectra were obtained on a Varian HA-100 spectrometer. Line positions were measured relative to the internal lock signal by counting the sweep oscillator frequency to the nearest 0.1 Hz.

Results and Discussion

A. Spectral Assignment. The observed spectrum of β-ψ (30°, pD 6.7, 0.12 *M*) is shown in Figure 2a. The ribose-ring hydrogen resonances were assigned initially by a comparison with uridine. The low-field doublet at 7.660 ppm was readily attributable to the H_6 proton of the pyrimidine base since the C_6 position is adjacent to a nitrogen atom. The small splitting of 0.8 Hz is due to a long-range (allylic) spin–spin coupling interaction with the $H_{1'}$ hydrogen. Such couplings are generally small (<3 Hz) and may be of either sign.[32] The presence of this coupling interaction between H_6 and $H_{1'}$ is additional evidence for the C–C attachment of the ribose to the base.

The resonance of the anomeric proton $H_{1'}$ comprises a quartet centered at 4.674 ppm. At 30° only the doublet centered at 4.649 ppm (splitting 0.8 Hz) is observed; that to lower field is obscured by the HDO resonance. The water peak could be shifted by increasing the temperature to reveal a second equally intense doublet with the same splitting. This assignment was established by irradiation[33] of the H_6 proton which removed the smaller (0.8 Hz) splitting and collapsed the quartet into a doublet separated by 5.0 Hz.

Successive irradiation of the band envelopes at 7.660, 4.674, 4.279, and 4.141 ppm under spin decoupling[33] or spin tickling[34] conditions permitted the assignment of these peaks to the H_6, $H_{1'}$, $H_{2'}$, and $H_{3'}$ protons, respectively, with the $H_{4'}$ proton comprising the envelope at 4.009 ppm. The remaining peaks at highest field could then be assigned to the $H_{5'}$ protons.

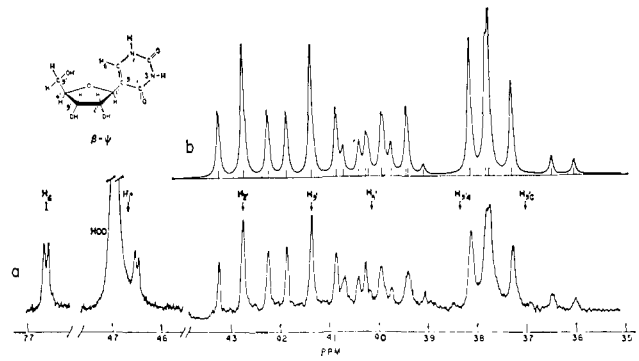

Figure 2. (a) Nmr spectrum at 100 MHz of β-pseudouridine in D_2O, pD = 6.7, 0.12 *M*, 30°. Chemical shifts are relative to internal 3-(trimethylsilyl)propanesulfonic acid. A second doublet of the quartet due to $H_{1'}$ is obscured by the HDO resonance at 4.7 ppm. (b) Computer-simulated nmr spectrum of the region due to ribose hydrogens 2′ through 5′.

Since the H_6 resonances were sufficiently separated from the other lines, the ribose protons could be computer-analyzed as a 6-spin system using a version of LAOCOON II[35,36] modified to give spin-tickling information.[37] The calculated spectrum shown in Figure 2b simulates the 2′–5′ region.

The chemical shift and coupling constant data are given in Tables I and II. For the sake of comparison, data for uridine taken from ref 15 are also included.

Table I. Proton Chemical Shifts of β-Pseudouridine and Uridine

	β-ψ[a,b]		U[b,c]
	30°	70°	28°
H_6	7.660	7.627	7.850[d]
$H_{1'}$	4.674	4.668	5.820
$H_{2'}$	4.279	4.260	4.260
$H_{3'}$	4.141	4.128	4.150
$H_{4'}$	4.009	3.993	4.060
$H_{5'B}$	3.840	3.835	3.825
$H_{5'C}$	3.726	3.719	3.735

[a] This work. [b] All shifts are given in parts per million and are positive values for resonance at low fields relative to internal DSS. [c] Reference 15. [d] B. J. Blackburn, unpublished results.

Table II. Coupling Constants of β-Pseudouridine and Uridine

	β-ψ,[a] Hz		U[b]
	30°	70°	28°
$J_{61'}$	0.8	0.8	<0.5
$J_{1'2'}$	5.0	5.2	4.4
$J_{2'3'}$	5.0	5.0	4.6
$J_{3'4'}$	5.2	5.2	4.9
$J_{4'5'B}$	3.2	3.4	
$J_{4'5'C}$	4.6	4.8	
$J_{5'B5'C}$	−12.7	−12.4	

[a] This work. [b] Reference 15.

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B. Establishment of the Anomeric Configuration by Comparison of the $H_{1'}$ Chemical Shifts. The application of nmr to this type of structural problem has met with some success.[38,39] Lemieux[38] was able to deduce the configuration of the naturally occurring deoxyribonucleoside thymidine by comparison of its proton resonance spectrum with that of its α anomer. In the β form $H_{1'}$ is *cis* to the 2'OH and *trans* to $H_{2'}$, whereas in the α form the reverse is true. In theory therefore one should be able to distinguish between the anomers on the basis of the $H_{1'}$ chemical shift and the magnitude of its coupling interaction with the $H_{2'}$ proton, $J_{1'2'}$. Application of coupling constant data to structural problems is generally based on the Karplus equation relating vicinal coupling constants, $J_{HH'}$, and the dihedral angle, ϕ, between the HCC' and CC'H' planes in the fragment HCC'H'.[40] The relationship was shown to have the form $J_{HH'} = J_0 \cos^2 \phi - 0.28$ Hz, where $J_0 = 8.5$ *for* $0° \leq \phi \leq 90°$ *and* 9.5 *for* $90° \leq \phi \leq 180°$. Though the general form has been substantiated by a number of workers,[32] Lemieux and Lineback[41] have pointed out some of its limitations. They concluded that in view of the range of possible dihedral angles (0–45° for *cis* hydrogens and 75–165° for *trans* hydrogens) the anomeric assignment can be considered definitive only when the vicinal coupling is less than 1 Hz. However, for pseudouridine $J_{1'2'}$ is 5.0 Hz in the β anomer, and 3.2 in the α anomer.[42] On this basis unambiguous assignment of the anomers of ψ is not possible *via* the Karplus relationship.

However, assignment of the anomeric species seems possible by comparison of the $H_{1'}$ chemical shifts. The $H_{1'}$ (β anomer) resonance is 0.323 ppm upfield relative to that of $H_{1'}$ (α anomer). A survey of[38,39,43,44] a wide variety of furanosides suggests that the differential shielding effects of a 2'-OH group are responsible for the observed differences (0.22–0.30 ppm) in resonance fields of the $H_{1'}$ protons of an anomeric pair. Thus, in general, a 2'-OH group with a *cis* relationship to a C–H bond in a flexible furanose system appears to exert a net shielding on the hydrogen, relative to the effect on a hydrogen situated in a *trans* position. There seems no *a priori* reason why a similar differential shielding mechanism should not be operative in the pseudouridines, and therefore we have assigned the anomer whose $H_{1'}$ resonances appear to higher field as β. We shall show in section C that the ribose conformations of pseudouridine and uridine are indeed very similar.

C. Conformation of the Furanose Ring. Although the Karplus relation may be of limited value in differentiating between anomeric species, it has been successful in predicting the geometry of a given riboside.[4,6,19,45,46] The dihedral angles between C–H bonds on adjacent carbons in a furanose ring are determined by the mode and extent of buckling of the ring. Thus a knowledge of the hydrogen coupling constants permits us to determine the stereochemistry of the ribose moiety. The approach has been hampered by poorly resolved spectra for the normal ribo- and deoxyribonucleosides and nucleotides. Owing to the inductive effects of $O_{1'}$ and N_1, the $C_{1'}$ position of these furanosides is relatively electron deficient. The anomeric resonances are therefore found considerably downfield from the other ribose peaks, and appear as simple doublets or quartets (often triplets) in ribose or deoxyribose derivatives, respectively. Hence approximate $J_{1'2'}$ values are readily obtained by a simple first-order treatment. A word of caution should be inserted here, however, for the $H_{1'}$ resonance of the ribosides is the X part of an ABX spectral system. Thus, the apparent two line spectrum is actually made up of four lines, two pairs of which are nearly degenerate. This often makes the widths of the $H_{1'}$ "doublet" considerably greater than the first-order lines due to H_5 and H_6. $J_{1'2'}$ is the separation between the two *strongest* lines of the four (only if $J_{1'3'} = 0$), and errors of up to 1 Hz in $J_{1'2'}$ can be made by measuring the separation between the *centers* of the two broad $H_{1'}$ lines.

The remaining ribose couplings are less easily extracted because of considerable overlap in the 2' to 5' hydrogen region. Consequently, deductions regarding furanose ring conformation in earlier studies have been based mainly on the approximate value of a single coupling constant from which a single dihedral angle, $\phi_{1'2'}$, may be estimated. Suggested conformations based on a single coupling constant should be accepted with some reservation and must be confirmed by measurement of the remaining coupling constants.

In this section we shall deduce the ribose conformations of β-ψ using the Karplus equation. The general form of this expression is thought to be correct. However, since vicinal coupling constants are known to depend not only upon the relevant dihedral angles, but also upon the nature of the other substituents on the H–C–C'–H' fragment (in particular their electronegativities[47]), the appropriate values of J_0 will depend on the system in question. Sternhell[32] has indicated that J_0 values may vary in the range from 8 to 16 Hz. For the present study we have chosen the values suggested by Abraham, *et al.*,[48] for a number of carbohydrate ring systems: $J_0 = 9.27$ Hz for $0° \leq \phi \leq 90°$ and $J_0 = 10.36$ Hz for $90° \leq \phi \leq 180°$.

Because of the $\cos^2 \phi$ term in the Karplus relation, two values of ϕ_{ij} are predicted for each value of J_{ij}, one less than 90° and one greater than 90°. At 30°, the observed values of $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ are 5.0, 5.0, and 5.2 Hz, respectively. Therefore $\phi_{1'2'}$, $\phi_{2'3'}$, and $\phi_{3'4'}$ are predicted to be approximately either 41 or 136°. However, values of $\phi_{1'2'}$ and $\phi_{3'4'}$ less than about 75° would require unreasonable buckling of the furanose ring and thus may be disregarded. Furthermore, values of $\phi_{2'3'}$ greater than about 60° need not be considered since they require the molecule to be severely strained by an unfavorable rotation about the $C_{2'}$–$C_{3'}$ bond. Thus, if the modified Karplus equation does account for the variation in the coupling constants, any molecular conformation proposed must account for the

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following apparent dihedral angles: $\phi_{1'2'} \approx \phi_{3'4'} \approx 136°$, and $\phi_{2'3'} \approx 41°$. *Any structure in which $H_{2'}$ and $H_{3'}$ are eclipsed (i.e., $\phi_{2'3'} = 0$) may be excluded outright* since the value of 9 Hz would be predicted for $J_{2'3'}$. These conformations are expected to be unstable since the bulky hydroxyl groups at the 2′ and 3′ positions would be eclipsed. In order that $\phi_{2'3'}$ be not equal to 0°, the ribose must be buckled in such a fashion that either the 2′- or 3′-carbon (or both) lies out of the plane defined by $C_{1'}$, $C_{4'}$ and $O_{1'}$. The buckled conformations which satisfy this requirement are illustrated in Figure 3. Table III contains ϕ_{ij}

Table III. Measured Dihedral Angles (ϕ) and Calculated Coupling Constants for Various Furanose Ring Conformations

Atom out of plane[a]	$\phi_{1'2'}$, deg	$J_{1'2'}$, Hz	$\phi_{2'3'}$, deg	$J_{2'3'}$, Hz	$\phi_{3'4'}$, deg	$J_{3'4'}$, Hz
$C_{2'}$ *endo*	165	9.5	45	4.3	105	0.4
$C_{3'}$ *exo*	145	6.7	40	5.0	100	0
$H_{2'}$, $H_{2'}$ eclipsed	120	2.3	0	9.0	120	2.3
$C_{3'}$ *endo*	105	0.4	45	4.3	165	9.5
$C_{2'}$ *exo*	100	0	40	5.0	145	6.7
Observed		5.0		5.0		5.2

[a] *endo* means the atom is located on the same side of the plane defined by $C_{1'}O_{1'}$ and $C_{4'}$ as the $C_{4'}$–$C_{5'}$ bond. *exo* means that it is found on the opposite side.

values estimated from molecular models and the corresponding predicted J_{ij} values. Clearly no one of the conventional conformations satisfies the second requirement that $\phi_{1'2'} \approx \phi_{3'4'} \approx 136°$. Examination of Dreiding models shows that this requirement can be satisfied *only if the molecule is not frozen in a given conformation but exists in an equilibrium between two or more buckled forms. This equilibrium must be such that during interconversion the substituents at the 2′ and 3′ positions pass through the eclipsed conformation.* The observed J_{ij} and the calculated ϕ_{ij} are therefore time averages, their magnitudes depending on the residence times of the forms in equilibrium. An additional restriction on the proposed equilibrium is that it be rapid on the nmr time scale since the entire spectrum of β-ψ can be accounted for by a unique set of coupling constants and chemical shifts. Several equilibria involving conventional forms and an eclipsed intermediate state can be visualized. The most attractive are[49] (a) a $C_{2'}$-*endo* ↔ $C_{3'}$-*endo* conversion requiring $\phi_{1'2'}$ and $\phi_{3'4'}$ to vary from 105 to 165°, and centered around 135° and predicting $0.4 < J_{1'2'} \approx J_{3'4'} < 9.5$ Hz and $4.3 < J_{2'3'} < 9.0$ Hz; (b) a $C_{2'}$-*exo* ↔ $C_{3'}$-*exo* conversion requiring $\phi_{1'2'}$ and $\phi_{3'4'}$ to vary in the range 100 to 145° and centered around 122° and predicting $0 < J_{1'2'} \approx J_{3'4'} < 6.7$ Hz and $5.0 < J_{2'3'} < 9.0$ Hz. Both possibilities are qualitatively in agreement with the present data. In view of the uncertainties involved in this treatment a choice between the two possibilities is not justified. X-Ray data on a variety of nucleosides and nucleotides[50,51] and polyadenylic acid[52] indicate that with few exceptions the furanose ring is buckled such that the displaced atom is in an *endo* position. Although the *endo* conformations appear to be favored in the solid state there is no *a priori* reason to assume that this is also the case for β-ψ in aqueous solution.

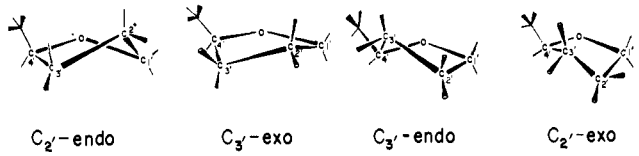

Figure 3. Possible buckled conformations for the ribose ring of β-pseudouridine.

A comparison of the data in Table II reveals a lack of any significant temperature dependence of $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$. Only slight increases in the coupling constants are observed at 70°, the largest being 0.2 Hz for $J_{1'2'}$. This implies that no significant changes in the ribose conformation occur over the temperature interval 30–70°. This is confirmed by the absence of any marked influence of temperature upon the chemical shifts, in particular that of the anomeric proton. Since the $H_{1'}$ hydrogen is shielded by the 2′-OH, any conformation change which alters $\phi_{1'2'}$ should be manifested in the $H_{1'}$ chemical shift. However in the temperature interval studied it was constant to within 0.006 ppm.

In view of the postulate of an equilibrium between ribose conformations, the absence of any temperature dependence of the coupling constants is strange. This can be expected if the energy difference between the forms in equilibrium is small. Space-filling models suggest that the distances between the various substituents on the ribose ring, in particular that between the 2′ and 3′ hydroxyl groups, are not markedly different in the $C_{2'}$-*endo* and $C_{3'}$-*endo* conformations. Therefore, nonbonded interactions are expected to be similar in the two forms and their energy differences small. (A similar argument can be applied to the *exo* forms.) Recent theoretical calculations by Flory and coworkers indicate that these differences are indeed small.[53]

It is of interest to compare the ribose coupling constant data for U and β-ψ (Table II). The differences in $J_{2'3'}$ and $J_{3'4'}$ are 0.4 and 0.3 Hz, respectively. Differences in J for 4′-5′$_B$, 4′-5′$_C$, and 5′$_B$-5′$_C$ could not be ascertained since the relevant parameters are not yet available for uridine. The relative inductive effects of the bases accounts for the different $J_{1'2'}$ values. A larger value of $J_{1'2'}$ in β-ψ is anticipated since examination of the variation of the vicinal coupling constant in a series of substituted ethanes indicates that substitution of a carbon for nitrogen could augment the vicinal coupling by as much as 0.5 Hz.[47] Therefore it seems likely that the smaller value of $J_{1'2'}$ in uridine is due primarily to the larger inductive effects of its aglycone rather than to any significant change in the apparent value of $\phi_{1'2'}$.

Since a reasonable correction for inductive effects makes $J_{1'2'}$ the same in U and β-ψ, and since the values

(49) An equilibrium involving structures in which both $C_{2'}$ and $C_{3'}$ lie out of the plane (but on opposite sides) defined by the remaining three atoms of the ring, *i.e.*, $C_{2'}$-*endo*, $C_{3'}$-*exo* ↔ $C_{2'}$-*exo*, $C_{3'}$-*endo*, is also reasonable. This equilibrium can not be discounted since it satisfies the above-mentioned requirements.

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T°C	P_I	P_{II}	P_{III}
30	0.53	0.32	0.15
70	0.47	0.35	0.18

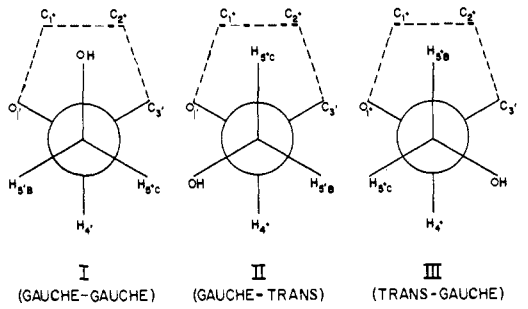

Figure 4. Rotational isomers around the $C_{4'}$–$C_{5'}$ bond of β-pseudouridine. The calculated relative populations of these isomers at 30 and 70° are shown above the structures.

of $J_{2'3'}$ and $J_{3'4'}$ differ by 0.4 Hz or less, we must conclude that the ribose conformations in U and β-ψ are similar. Thus, a rapid equilibrium best describes the structure of the ribose in U. Fujiwara[15] has concluded from his coupling data for uridine (Table II) that an equilibrium between two puckered ribose forms is not likely and that the actual conformation is *either* $C_{2'}$-*endo* or $C_{2'}$-*exo*. Assuming the applicability of the Karplus equation, $\phi_{1'2'}$ or $\phi_{3'4'}$ should be about 50°. The required angle however lies 25° to the low side of the range of values for *trans*-hydrogens on adjacent carbons in a five-membered ring[41] and suggests a highly strained structure. Recently Prestegard and Chan[5] concluded, solely on the bases of $J_{1'2'}$, that an average conformation intermediate between $C_{2'}$-*endo* and $C_{3'}$-*endo* best describes the ribose conformation of U and did not discount the possibility of an equilibrium between two forms.

D. Conformation of the Exocyclic CH_2OH Group. In recent years considerable research has been devoted to the investigation of the forces stabilizing the conformation of polynucleotides. Nonbonded base-stacking interactions have been foremost among those studied and they appear to be of primary importance in maintaining the purine and pyrimidine bases in planar stacked configurations. These forces alone, however, cannot account for the conformational rigidity of the ribose–phosphate backbone. The backbone of polynucleotides consists of furanoside rings linked *via* $C_{4'}CH_2OPOC_{3'}$ fragments. The conformational preference of the backbone is no doubt due in part to rotational barriers in the five exocyclic single bonds ($C_{4'}$–$C_{5'}$, $C_{5'}$–$O_{5'}$, $O_{5'}$–P, P–$O_{3'}$ and $O_{3'}$–$C_{3'}$). Because of the sensitivity of proton–proton and proton–phosphorus coupling constants to the dihedral angle in $HC_{4'}C_{5'}H$ and $H_{5'}C_{5'}OP$ fragments, respectively, nmr should be of considerable use in determining preferred backbone conformations. Some attempts have been made to determine conformations of nucleotides in solution by measurement of $H_{5'}P$ coupling constants.[16,17] However, no detailed investigation of the coupling interaction between the $H_{4'}$ and $H_{5'}$ hydrogens with regard to the stereochemistry of the $C_{4'}$–$C_{5'}$ bond has been undertaken. It would be useful then to see whether the value of $J_{4'5'}$ can yield information regarding preferred conformation of the $C_{4'}$–$C_{5'}$ bond of β-ψ.

The three classical, staggered rotamers about the $C_{4'}$–$C_{5'}$ bond are shown in Figure 4. Since the 4′-carbon is asymmetric, the rotational isomers differ in energy and for a rapid interconversion the 4′-5′ coupling constants are an average over the residence times of the molecule in each of the rotamers. Assuming a set of staggered rotamer configurations one can calculate their relative populations from

$$J_{4'5'B} = P_I J_{gI} + P_{II} J_{gII} + P_{III} J_{tIII} \quad (1)$$

$$J_{4'5'C} = P_I J_{gI} + P_{II} J_{tII} + P_{III} J_{gIII} \quad (2)$$

where J_g and J_t are the *gauche* and *trans* coupling constants and P_I, P_{II}, and P_{III} are the mole fractions of each rotamer. These equations reduce to

$$J_{4'5'B} = 2.0(P_I + P_{II}) + 10.1P_{III} \text{ and } J_{4'5'C} = 2.0(P_I + P_{III}) + 10.1P_{II} \quad (3)$$

if the *gauche* and *trans* value do not vary among the rotamers and can be accounted for by the Karplus relation.

The resonances of the 4′ and 5′ hydrogens lie in the region from 3.60 to 4.10 ppm (Figure 1). It is clear from the appearance of this region that the two 5′-hydrogens are magnetically nonequivalent and comprise, with $H_{4'}$, an ABC spectral subsystem. The magnetic nonequivalence of methylene hydrogens in ethanelike molecules of the type CH_2XCHYZ has been studied extensively[54] and discussed in terms of a temperature-dependent contribution due to unequal rotamer populations and a temperature-independent contribution due to intrinsic molecular asymmetry. At 30°, the 5′ hydrogens are chemically shifted by 0.116 ppm; at 70° their relative shielding is reduced to 0.108 ppm; presumably the result of partial equalization of the rotamer population (Figure 4). At 30° the vicinal coupling constants $J_{4'5'B}$ and $J_{4'5'C}$ were found to be 3.2 and 4.6 Hz, respectively. This inequality indicates that rotamers I, II, and III are not equally populated. In the high temperature limit where $P_I = P_{II} = P_{III} = {}^1/_3$, $J_{4'5'B}$ and $J_{4'5'C}$ become identical and equal to ${}^1/_3(J_t + 2J_g)$. At 70°, the vicinal couplings are 3.4 and 4.8 Hz indicating that only slight changes in rotamer population are occurring.

Using the measured values of $J_{4'5'B}$ and $J_{4'5'C}$, we have calculated from eq 3 the relative populations of the three rotamers (Figure 4). The data in Figure 4 suggest that the dominant form is I (*gauche–gauche*) in which the 5′-OH is *gauche* to both $C_{3'}$ and $O_{3'}$. In this rotamer the hydroxyl group is above the plane of the ribose ring and at its distance of closest approach to the uracil base. Rotamers II and III, in which the 5′-OH group lies off the ring at a greater distance from the uracil base, appear to be present to a lesser extent.[55] At 70°, the calculation indicates that the *gauche–gauche* rotamer still predominates.

(54) See review by M. Van Gorkom and G. E. Hall, *Quart. Rev.* (London), 22, 14 (1968).

(55) It should be emphasized that the individual 5′-CH_2 hydrogen resonances cannot be assigned to a particular position in a given rotamer (Figure 4). Interchanging the assignments of the 5′-B and 5′-C hydrogens serves to interchange the calculated populations of rotamers II and III, but has no effect on the conclusion that the *gauche–gauche* rotamer is preferred.

It is interesting to compare our calculations with structures obtained from X-ray diffraction data. Although the crystal structure of β-ψ is not known, data on a wide variety of purine and pyrimidine nucleosides and nucleotides indicate that the *gauche–gauche* conformation occurs most frequently.[50] This is also the conformation observed in polyadenylic acid[52] and DNA in the solid state.[56]

The assumption that the classical staggered forms are minima of potential energy is strictly valid only for symetrically substituted C–C fragments, *i.e.*, CH_3CH_3 and CH_3CY_3. The X-ray data for adenosine-5′-phosphate, cytidine-3′-phosphate, deoxyadenosine, and 5-fluorodeoxyadenosine indicate that repulsion between $O_{5'}$ and $O_{1'}$ results in a larger angle between them in rotamers I and II; a similar repulsion between $O_{5'}$ and $O_{3'}$ also apparently increases the angle between them in rotamer III.[50,57] We have, therefore, calculated the populations of the rotamers derived from I, II, and III by allowing excess angles of 15° due to oxygen–oxygen repulsions. The resultant populations are $P_{I'} = 0.38$, $P_{II'} = 0.30$, $P_{III'} = 0.32$. Thus, there is still a preference for the *gauche–gauche* rotamer, but the preference is less than that calculated from the classical rotamer structures. The calculations with and without repulsions of 15° can be considered as extreme cases, and therefore the actual rotamer populations probably lie somewhere between the two sets. A preference for the *gauche–gauche* rotamer is thus still indicated.

E. The Sugar-Base Torsion Angle. The question of the relative orientations of the planar purine and pyrimidine rings with respect to the sugar as determined by rotation about the glycosyl bond has received considerable attention.[3,6,52,58] To facilitate discussion of this question the sugar-base torsion angle (ϕ_{CN}) has been defined.[58] X-Ray data indicate that most purine and pyrimidine nucleosides and nucleotides are in the *anti* range of ϕ_{CN} in the solid state.[50] Haschmeyer and Rich[51] have shown by consideration of close contact interaction between the base and ribose moieties that the *syn* conformation in pyrimidine nucleosides is unlikely. ORD measurements[59–61] suggest that the *anti* conformation is retained in solution. In addition, recent studies[3,6] on the pH-dependent effects of phosphate on the proton chemical shifts support the existence of the *anti* conformation in purine and pyrimidine 5′-mononucleotides. Comparison of the molecular models of uridine and pseudouridine show that the 2′ and 3′ positions of the ribose ring come in close contact with the base and suggest that similar *syn* and *anti* ranges exist in the sugar-base torsion angle (henceforth referred to as ϕ_{CC} in pseudouridine). Figure 5 illustrates the *syn* and *anti* conformations of β-ψ. In the *anti* conformer the C_6-carbon lies over the ribose ring; in the *syn* the C_4-keto group lies above the ribose and bears a spatial relationship with it similar to that of the 2-keto group in uridine. To explore this point

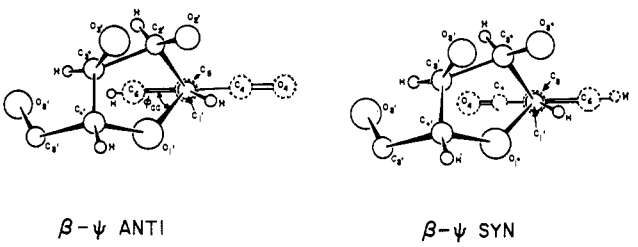

Figure 5. Possible isomers around the glycosidic bond of β-pseudouridine. The terms *syn* and *anti* are analogous to those used for uridine.[58]

using the present data, we shall discuss the data in two sections; (i) chemical shifts and (ii) the $J_{61'}$ allylic coupling constant.

(i) **Chemical Shifts.** As previously mentioned, a striking similarity exists between the corresponding furanose chemical shifts for U and β-ψ. In view of the substantial anisotropic effect of the keto group[62] and its proximity to the $C_{1'}$, $C_{3'}$, and $C_{5'}$ positions in the *syn* conformation, one can only conclude that the keto group has a similar location in both molecules, *i.e.*, that β-ψ also exists in the *anti* conformation. ORD measurements suggest that β-ψ exists in solution as the *anti* form,[61] but the curves were ill-defined and the interpretation inconclusive.

The occurrence of the H_6 resonance of β-ψ 0.20 ppm to low field of its counterpart in U is perhaps surprising if their conformations are similar. Prestegard and Chan[5] rationalize the 0.34 ppm downfield shift of the H_6 hydrogen of uracil upon substitution of a ribose at the N_1 position on the basis of the through-space electric field effect of the ether oxygen. If the base conformation in β-ψ is *anti* the ether oxygen effect must still be reflected in the H_6 shift except that it is apparently masked by an opposing substituent effect.[63] Substitution of a methyl group at the 5 position of uracil causes a displacement of the H_6 resonance 0.13 ppm to high field.[30] Thus a gross conformation change need not be invoked to rationalize the upfield shift of the H_6 hydrogen of β-ψ.

(ii) $J_{61'}$ **Allylic Coupling.** The allylic coupling in β-ψ should provide information regarding the sugar-base torsion angle. It has been demonstrated that allylic couplings are related to the dihedral angles between the relevant CCH planes,[32] and can vary between ±3 Hz. Concomitant with a change in the sugar-base torsion angle ϕ_{CC} is a change in the dihedral angle between the $H_6C_6C_5$ and $C_5C_{1'}H_{1'}$ planes, which would be manifest in $J_{61'}$. For example, whereas in β-ψ at 30° this coupling is 0.8 Hz, it is 1.2 Hz in the α isomer.[42] Since the electronic environments are probably the same in each case, this variation must reflect the difference in ϕ_{CC} for the two anomers.

The minimum magnitudes of the allylic couplings are expected to occur for dihedral angles of 0 or 180°.[32] These correspond to the *syn* and *anti* conformations, respectively. Thus, the small magnitude of $J_{61'}$ demonstrates only that ϕ_{CC} lies in one of the accepted ranges, and we cannot use it to distinguish between them. However, variation in ϕ_{CC} due to solvent or temperature perturbations should be readily detected by changes in this coupling. We find that $J_{61'}$ is constant over the

(56) W. Fuller, M. H. F. Wilkins, H. R. Wilson, and L. D. Hamilton, *J. Mol. Biol.*, 12, 60 (1965).
(57) We are grateful to Professor R. U. Lemieux for a very helpful discussion on this phenomenon.
(58) J. Donahue and K. N. Trueblood, *J. Mol. Biol.*, 2, 363 (1960).
(59) T. L. V. Ulbricht, J. P. Jennings, P. M. Scopes, and W. Klyne, *Tetrahedron Lett.*, 695 (1964).
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(61) T. R. Emerson, R. J. Swan, and T. L. V. Ulbricht, *Biochemistry*, 6, 843 (1967).
(62) A. A. Bothner-By, *Ann. Rev. Phys. Chem.*, 16, 43 (1965).
(63) We thank the referee for this suggestion.

Table IV. Effect of Purine on β-ψ Chemical Shifts

X	Δ_x β-ψ, ppm	Δ_x uridine, ppm
H_5		0.32
H_6	0.176	0.24
$H_{1'}$	0.231	0.22
$H_{2'}$	0.125	0.090
$H_{3'}$	0.076	0.083
$H_{4'}$	0.060	0.081
$H_{5'B}$	0.049	0.066
$H_{5'C}$	0.054	0.087

temperature range 5–70°, demonstrating that no change occurs in the sugar-base torsion angles.

F. Effect of Purine on the Chemical Shifts of β-ψ. In Table IV are listed Δ_x values for β-ψ at 30°. Δ_x has been defined as the chemical shift of a given hydrogen in a 0.12 *M* solution relative to its resonance position in the presence of 1 *M* purine; Δ_x is taken to be positive if the addition of purine results in a displacement of the hydrogen resonance to higher magnetic field. Also given in Table IV are the Δ_x values for a 0.11 *M* aqueous solution of uridine at 35° obtained by Schweizer, *et al.*[30] A striking similarity can be seen in the magnitudes of Δ_x values of corresponding hydrogens. In all instances the values are positive indicating that the hydrogens move upfield upon addition of purine to the solution. Their magnitudes are largest for the base and $H_{1'}$ hydrogens and decrease progressively around the ribose ring with increasing distance from the glycosidic bond and are a minimum for the 5′ methylene hydrogens. Schweizer, *et al.*,[30] have considered the effects of purine on the chemical shifts of U in terms of intermolecular complex formation. The direction of the purine-induced shifts and their variation with distance from the glycosidic bond suggested that the interaction is that of vertical stacking of the purine and pyrimidine bases. In view of the similarity in the trends in Δ_x we suggest that analogous base-stacked complexes of β-ψ are formed in aqueous solution. It is interesting that in the present study the coupling constants of β-ψ are not significantly affected by the addition of purine. The changes are less than 0.2 Hz in all of the observed values. It would appear from this observation that the conformation of the ribose (including the exocyclic rotamer population) is not affected. The constancy of $J_{61'}$ also suggests that on formation of a stacked complex in 1 *M* purine the sugar-base torsion angle remains constant.

Conclusion

The conformations of β-pseudouridine and uridine in aqueous solution are similar, and essentially independent of temperature over the range 30–70°. If one is to explain the unique occurrence of β-ψ in some regions of transfer RNA structure, it must then be in terms of the extra potential hydrogen bond that can be formed by the N–H at position 1 in β-ψ.

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