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OLIGONUCLEOTIDE CONFORMATIONS.
(5) NMR and relaxation studies on GpU and UpG at neutral pH[‡]

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The average conformation of GpU and UpG in neutral aqueous solutions has been investigated by proton chemical shifts and coupling measurements as well as by T_1 relaxation time experiments. The proportion of the N and S pseudorotational conformers of the ribose ring has been derived from the vicinal coupling constants.

The relaxation data provide information about the *syn–anti* equilibrium of the orientation of the base about the glycosidic bond. This orientation is predominantly *syn* for the Guo base in both dinucleoside phosphates, that of Urd is *anti* in the case of GpU and shows an almost equivalent *syn* and *anti* character for UpG.

1. Introduction

The study of dinucleoside phosphates has been initiated in order to obtain information on the structure and conformation of polynucleotides and nucleic acids, the dinucleoside phosphates being regarded as the simplest case of a single-stranded helix. This supposition seems to hold for homo-dinucleoside phosphates, and holds best for ApA [1,2].

But even in the first optical studies [1–3], it became apparent that certain dinucleoside phosphates containing uridine in either the 3′ or 5′ position were only slightly stacked at room temperature. This led to the suggestion that uridine had an unstacking influence [4], especially if next to a purine base. One dinucleoside phosphate only showed stacking upon protonation: GpU [2]. An unusual perpendicular structure was proposed for GpU at neutral pH [5] and a stacked structure in the acid form, with guanosine in the *syn* conformation in both structures, contrary to the generally accepted idea that in dinucleoside phosphates the bases were oriented *anti*, as in the nucleic acid.

The recent availability of relaxation techniques [6–13] has modified many concepts on nucleoside conformations and has quite clearly shown that *all* nucleosides can assume the *anti* and the *syn* orientation of their bases. In this report we have studied GpU and UpG by proton–proton spin–lattice relaxation and it is shown that this *syn–anti* equilibrium also exists in these dinucleoside phosphates.

2. Material and methods

GpU and UpG were purchased from P.L. Laboratories. Centimolar solutions were prepared in D_2O and lyophilized three times, the pD was adjusted to about 7.5 and the solutions freed from bivalent ions by treatment with Chelex 100. Measurements were performed on a CAMECA 250 MHz Spectrometer in the Fourier transform mode. The T_1's were determined at 42 ± 1°C by the inversion recovery method (180° t, 90° pulse sequence). Deuteration of H^8 of Guo was obtained by heating for 3 h to 80° in D_2O. The samples were freed from oxygen by degassing and sealed under nitrogen atmosphere.

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2.1. Proton relaxation experiments

The *initial* time dependence of the longitudinal magnetization of a proton following a 180° pulse is given by

$$M_0 - M(t) = 2M_0 \exp(-t/T_1), \tag{1}$$

where M_0 is the equilibrium value of the magnetization and T_1 the longitudinal relaxation time. In the case of a proton H_i relaxed only by dipolar interaction with an ensemble of protons j, T_{1i} is given by

$$T_{1i}^{-1} = \frac{3}{10}\gamma_H^4 \hbar^2 f(\tau_c) \sum_j \langle r_{ij}^{-6}\rangle, \tag{2}$$

with

$$f(\tau_c) = \tau_c/(1+\omega^2\tau_c^2) + 4\tau_c/(1+4\omega^2\tau_c^2),$$

γ_H is the proton gyromagnetic ratio, ω the Larmor frequency, τ_c the correlation time which in the case of nucleosides and nucleotides [11–13] has been shown to be that of the isotropic reorientation of the whole molecule, and $\langle r_{ij}^{-6}\rangle^{-1/6}$ the distance of H_i to any H_j of the molecule, averaged by internal motions. In extreme narrowing conditions eq. (2) may be replaced by

$$T_{1i}^{-1} = \frac{3}{2}\gamma_H^4 \hbar^2 \sum_j \langle r_{ij}^{-6}\rangle \tau_c. \tag{3}$$

Because of the cross relaxation in this multispin system, it is expected that the semi-logarithmic plot of $M_0 - M(t)_i$ versus time departs progressively from linearity. In the case of nucleosides and nucleotides this effect has been discussed by Akasaka et al. [13]. From the curves computed by these authors for a three-spin system like $H_{1'}$, $H_{2'}$ and H^8 it may be estimated for instance that in the time interval $0 < t < 0.7\ T_1$ the error introduced by neglecting the cross relaxation in the measurement of T_1 does not exceed ca. 12% for the most unfavourable geometrical arrangement of these protons. In the present case however, we have observed that eq. (1) remains valid within the limits of experimental errors (± 5%) at least in the interval $0 < t < 1.5\ T_1$ so that the T_{1i}'s were merely obtained from the initial slope of log $(M_0 - M(t)_i)$.

The relaxation of all protons of the molecules under study being governed by the same correlation time τ_c, it is seen from eq. (3) that their average conformation may be obtained as shown previously [11] by seeking the minimum standard deviation $\Delta\tau_c/\bar{\tau}_c$ about the mean $\bar{\tau}_c$ of the $(\tau_c)_i$ calculated from the experimental T_{1i}'s and computed interproton distances. In our experimental conditions where τ_c is of the order of 10^{-10}s the extreme narrowing condition is not exactly fulfilled at $\omega = 1.57 \times 10^9$ rad s^{-1} so that we have chosen $\Delta f(\tau_c)/\overline{f(\tau_c)}$ as an agreement factor instead of $\Delta\tau_c/\bar{\tau}_c$, which leads to equivalent results but needs slightly more complicated calculations.

The term $\Sigma_j\langle r_{ij}^{-6}\rangle$ needed for the calculation of $\Delta f(\tau_c)/\overline{f(\tau_c)}$ from eq. (2) was obtained by computing as a function of the torsion angle Υ about the glycosidic bond ($\Upsilon = \varphi - 120°$), the minus sixth power of the interproton distances weighted by the fractions n and s of the pseudorotational conformers N and S of the ribose ring and by the respective probabilities P_{gg}, P_{gt} and P_{tg} of the gauche–gauche, gauche–trans and trans–gauche rotamers of the exocyclic methylene group. n and s as well as the pseudorotational ^NP and ^SP and the maximum torsion angle τ_m which give the geometry of the ribose ring are derived from the vicinal coupling constants [14]. The values of these parameters obtained by a graphical method [15] are given in table 3. Since the coupling constants between protons 4', 5' and 5'' are not always available because of overlap, reasonable values of rotamer distribution of the exocyclic group $P_{gg} = 0.7$, $P_{gt} = P_{tg} = 0.15$ were used in all calculations. The fixed interatomic distances were taken from Saenger's review [16] and the C—H distance used in our calculations was 0.1085 nm.

The plots of $\Delta f(\tau_c)/\overline{f(\tau_c)}$ versus Υ generally show two pronounced minima at angles Υ_I and Υ_{II} which are always located in the *syn* ($-70° < \Upsilon < 110°$) and *anti* ($110° < \Upsilon < 290°$) ranges, respectively (fig. 3a and 4a). These two minima correspond to the preferential orientations of the base about the glycosidic linkage [11].

Their probability is obtained by searching for the weighted average of the $\Sigma_j\langle r_{ij}^{-6}\rangle$ calculated for Υ_I and Υ_{II} which gives a new minimum of $\Delta f(\tau_c)/\overline{f(\tau_c)}$ smaller than two previous ones (fig. 3b and 4b).

We can now determine the average distance between H^8 of Guo or H^6 of Urd, respectively and the corresponding ribose protons. These distances can also be evaluated from the increase in relaxation time T_1 of the ribose protons after deuteration of H^8 of Guo:

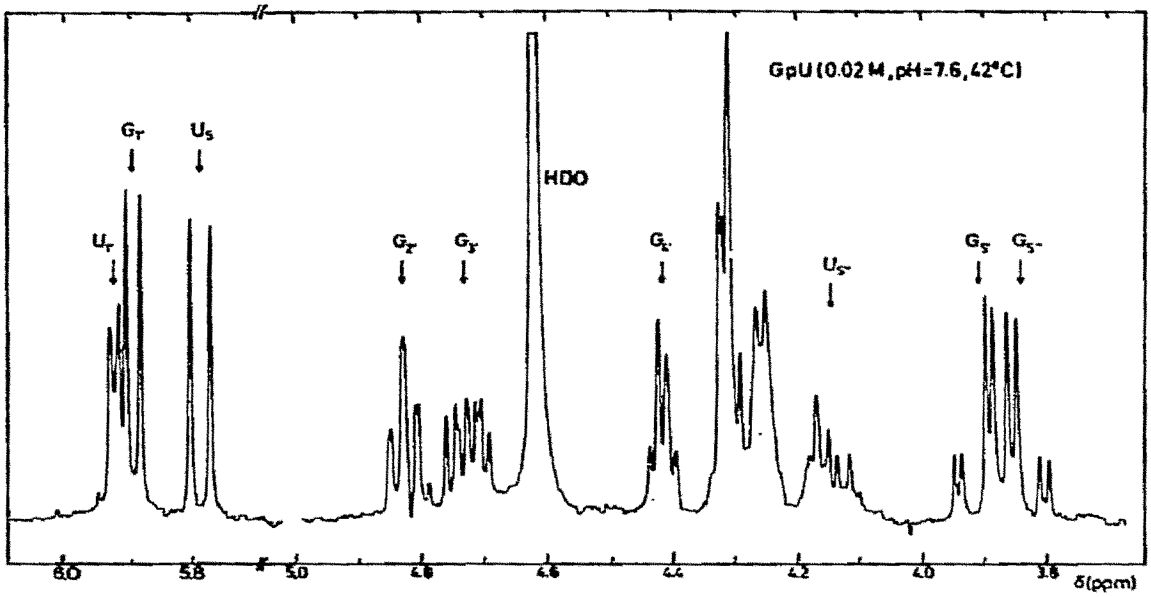

Fig. 1. High resolution PMR spectrum of GpU at 42°C.

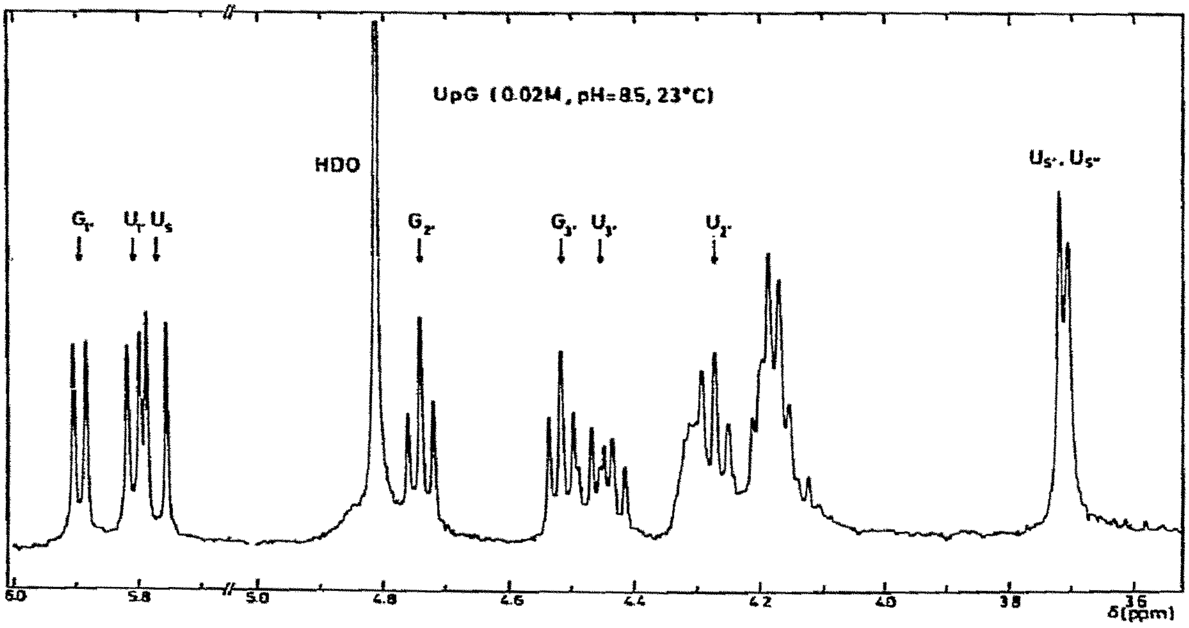

Fig. 2. High resolution PMR spectrum of UpG at 23°C.

Table 1
Chemical shifts (ppm/DSS, $T = 42°C$)

	Guo moiety (Gp, pG)							Urd moiety (Up, pU)							
	G_8	$G_{1'}$	$G_{2'}$	$G_{3'}$	$G_{4'}$	$G_{5'}$	$G_{5''}$	U_6	U_5	$U_{1'}$	$U_{2'}$	$U_{3'}$	$U_{4'}$	$U_{5'}$	$U_{5''}$
GpU	7.940	5.945	4.827	4.774	4.416	3.907	3.836	7.801	5.841	5.926		4.41	4.25		4.123
3'GMP	8.004	5.918	4.765	4.724	4.369	3.906	3.886								
5'UMP [17]								8.108	5.972	5.981	4.413	4.340	4.254	4.017	3.961
Δδ	−0.064	0.027	0.062	0.050	−0.047	0.001	−0.050	−0.307	−0.132	−0.055					0.172
UpG	8.025	5.896	4.740	4.510	4.286	4.175	4.180	7.742	5.776	5.811	4.272	4.457	4.174	3.713	3.710
3'UMP [18]							4.008	7.890	5.890	5.927	4.395	4.484	4.227	3.893	3.852
5'GMP	8.179	5.903	4.739	4.496	4.329	4.031									
Δδ	−0.154	−0.007	0.001	0.023	−0.043	0.144	0.172	−0.148	−0.014	−0.116	−0.123	−0.027	−0.053	−0.175	−0.142

Table 2
Coupling constants (Hz)

	Guo moiety (Gp, pG)								Urd moiety (Up, pU)							
	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{5'5''}$	$J_{3'p}$	$J_{2'p}$	$J_{5\text{-}6}$	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'\text{-}5'}$	$J_{4'\text{-}5''}$	$J_{5'5''}$	$J_{5''p}$
GpU (23°C)	5.3	5.3	3.8	2.7	3.6	−13.0	8.1	1.1	8.0	3.8				2.7	−11.2	4.5
(42°C)	5.7	5.2	3.7	2.9	3.8	−13.0	7.9	1.2	8.0	3.6				2.5	−11.2	4.7
3'GMP (40°C)	5.7	5.2	3.4	3.5	3.8	−12.7	7.5	0.3								
5'UMP [17]										5.1	4.8	4.1	2.3	2.8	−11.8	
UpG (23°C)	5.0	5.2	4.7						8.0	5.0	4.9	5.3				$J_{3'p} = 8.2$ $J_{2'p} = 1.0$
3'UMP [18]										4.2	4.8	5.4	2.8	3.8	−12.8	
5'GMP (23°C)	5.8	5.1	3.8	3.6	3.7	−11.6										

$$(1/T_1)_j^{\mathrm{H}} - (1/T_1)_j^{\mathrm{D}} = \tfrac{3}{10}\, \gamma_{\mathrm{H}}^4 \hbar^2 f(\tau_{\mathrm{c}}) \langle r_{8j}^{-6} \rangle, \qquad (4)$$

where $f(\tau_c)$ is again given by the minimum of $\Delta f(\tau_c)/\overline{f(\tau_c)}$.

3. Results

3.1. Spectral analysis

Identification of the base protons is straightforward; H^8 of Guo is a singlet, H^6 and H^5 are doublets with $J_{56} = 8$ Hz as in UMP [16,17]. In fig. 1 the ribose proton spectrum of GpU in aqueous neutral solution at 42°C is shown. This temperature was chosen, because at room temperature a part of the spectrum is hidden by the HDO peak. Protons $H_{5'}$ and $H_{5''}$ of Guo are more upfield and form an octuplet because of their coupling with $H_{4'}$. Comparison with the Guo-3'-P spectrum [19] permitted easy assignment of $H_{4'}$, $H_{3'}$ and $H_{2'}$. Homonuclear decoupling confirmed these assignments. The Urd ribose protons are poorly resolved, and only $H_{1'}$ and $H_{5''}$ are well separated.

In fig. 2 the spectrum of the ribose protons of UpG at 23°C is shown. Most of the proton resonances are well resolved, only $H_{4'}$ of Urd and $H_{4'}$, $H_{5'}$ and $H_{5''}$ of Guo are together in a group between 4.1 and 4.3 ppm.

The chemical shifts of GpU and UpG are summarized in table 1 and are compared with the corresponding 3' and 5' nucleotides.

For the Guo moieties of both dinucleoside phosphates no large variations in chemical shifts were observed, compared with the component nucleotides. Only the H^8 of UpG is shifted upfield probably due to the ring current of the neighbouring uracil ring. All other dimerization shifts of Guo are smaller than 0.07 ppm. In contrast, some dimerization shifts in the Urd moiety are larger. H^5 and H^6 are both shifted upfield. Also, it should be noted that $H_{1'}$ and H^5 of Urd are quite well separated in GpU (fig. 1), but much closer in UpG (fig. 2), contrary to the corresponding mononucleotides [17,18]. The influence of the ring current of the Guo base upon the aromatic protons of Urd in GpU and a similar effect of Urd on H^8 of Guo in UpG are responsible for these upfield shifts. Similar conclusions were already reached by Ts'o et al. [20].

The proton–proton and proton–phosphorus coupling constants of UpG and GpU are summarized in table 2 and compared with those of the component nucleotides. In GpU the coupling constants of Guo are very close to those of Guo-3'-P, except $J_{4'5'}$, which is considerably smaller. J_{PH} couplings are smaller, however. In UpG the ribose coupling constants show parallel shifts, $J_{1'2'}$ decreases and $J_{3'4'}$ increases, compared with Guo-5'-P indicating a slight shift towards a more predominant N-conformer [15] (see below). In the Urd moieties the coupling constants are quite difficult to extract and different in both cases from those of the corresponding nucleotides [17,18]. In UpG, $J_{1'2'}$ increases compared with Urd-3'-P, but $J_{3'4'}$ does not change very much. In GpU, $J_{1'2'}$ decreases by 1.5 Hz compared with Urd-5'-P, while $J_{3'4'}$ is quite large, indicating a predominant N conformation (see below). These coupling constants are quite similar to those of ApU and its analogs [21,22].

3.2. Ribose conformation and exocyclic rotamers

In table 3 the conformational parameters of the sugar moieties and the exocyclic rotamer distributions are listed. The sugar conformer distribution depends on the base and the position of the phosphodiester group. The S form predominates in Guo, while N predominates in Urd. Also the N conformer contribution is more important in the case of the 5'-phosphates (pG, pU) than

Table 3
Conformational parameters of sugar moieties of GpU and UpG

	Nucleoside	N_P	S_P	τ_m	%N	%S	P_{gg}	P_{gt}	P_{tg}
GpU	GuO	18°	172°	38°	39.4	60.6	0.67	0.22	0.11
42°	Urd	−15°	195°	44°	58.8	41.2			
UpG	Guo	6°	174°	40°	49.5	50.5			
23°	Urd	10°	170°	43°	51.5	48.8			

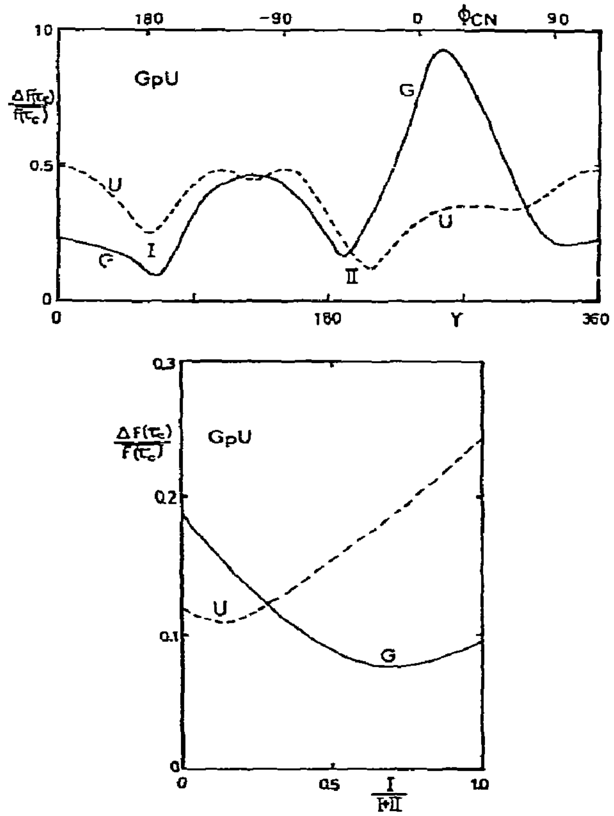

Fig. 3. (a) Determination of τ_I and τ_{II} from the minimum standard deviation around $\overline{f(\tau_c)}$ for GpU. $\overline{f(\tau_c)}$ has been deduced from the relaxation of H^8 (or H^6, respectively) by the corresponding sugar protons (see also table 4). (b) Determination of fraction of τ_I from the minimum standard deviation around $\overline{f(\tau_c)}$ of GpU. —— Guo moiety, - - - Urd moiety.

in the 3′-phosphates (Gp, Up). The more structured UpG [2] shows about equal proportions of N and S in both nucleoside moieties, while in the unstacked GpU [2] a large divergence of the conformers is observed between the two nucleosides. Also lowering the temperature appears to increase the N form contribution. These results suggest that oligonucleotides of larger chain length, where each nucleoside is linked to a 3′ and a 5′ phosphate, should show a preponderance of the N conformation, as it had been observed in RNA by X-ray fiber diffraction [23].

Unfortunately, the only exocyclic rotamer distribution of Guo in GpU is attainable and shows a 67% *gauche–gauche* conformation. Increasing the temperature from 22°C to 42°C decreases the P_{gg} and the difference between $H_{5'}$ and $H_{5''}$ ($\Delta\delta_{5'5''}$), as it had been observed previously in nucleosides and nucleotides [19]. In the case of pU, although we cannot compute exactly the rotamer distribution, the small value of $J_{4'5''}$ = 2.7 Hz indicates that P_{gg} is here preponderant too.

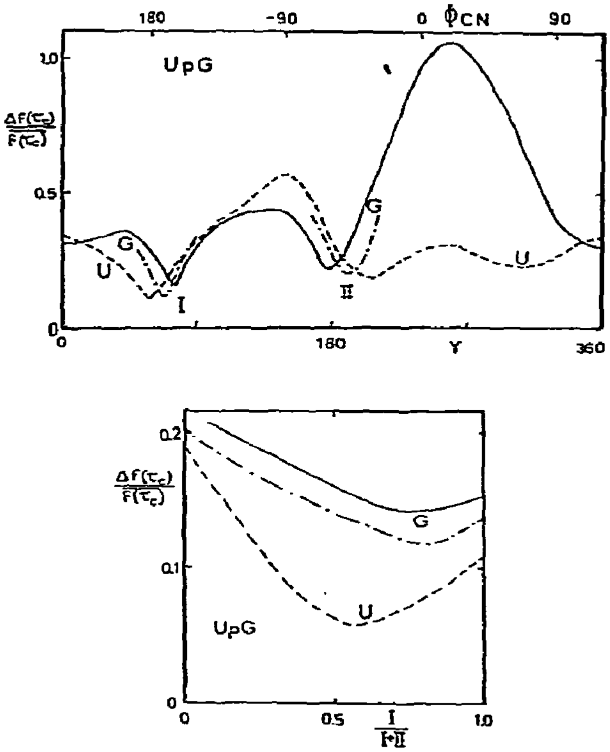

Fig. 4. Same experiment as in fig. 3 for UpG: —— Guo moiety, - - - Urd moiety, — - — - — Guo moiety, also taking into account the relaxation of H^8 by $H_{3'}$ of Urd.

3.3. Spin–lattice relaxation

In fig. 3 and table 4 the relaxation results on GpU are summarized. One notes in fig. 3a that both Guo and Urd can assume the two conformers *anti* and *syn*, corresponding to the minima I and II, respectively. These minima are separated by large barriers. If the agreement factor, $\Delta f(\tau_c)/\overline{f(\tau_c)}$ is plotted against the mole fraction of *syn* conformation [I/(I + II)], a very neat distribution curve is obtained; the minimum indicates the proportion of either of the two conformers (fig. 3b). In GpU, Guo has a large preference for the *syn* conformation (75%), while the Urd moiety is 85% of

Table 4
Proton relaxation data

GpU									
			H_8	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$	
Gp	T_1 (s)		2.02	2.28	1.06	1.00	1.44	0.35	
	T_1 (s)		(D_8)	3.40	1.45	1.10	1.35	0.37	
			$\Upsilon_{\mathrm{I}} = 66°, P_{\mathrm{syn}} = 0.75$,		$\Upsilon_{\mathrm{II}} = 190°, P_{\mathrm{anti}} = 0.25$				
			$\overline{f(\tau_c)} = 5.43 \times 10^{-10}$ s,		$\tau_c = 1.2 \times 10^{-10}$ s				
			j =	1'	2'	3'	4'	5'5"	
	$\langle r_{8j}^{-6}\rangle^{-1/6}$ (nm)	a		0.298	0.272	0.348	0.514	0.380	
		b		0.294	0.267				
pU				H_6	H_5	$H_{1'}$	$H_{5'}$		
	T_1 (s)			1.1	2.95	3.12	0.3		
			$\Upsilon_{\mathrm{I}} = 60°, P_{\mathrm{syn}} = 0.15$,		$\Upsilon_{\mathrm{II}} = 210°, P_{\mathrm{anti}} = 0.85$				
			$\overline{f(\tau_c)} = 4.4 \times 10^{-10}$ s,		$\tau_c = 9.5 \times 10^{-11}$ s				
			j =	5	1'	2'	3'	4'	5'5"
	$\langle r_{6j}^{-6}\rangle^{-1/6}$ (nm)			0.241	0.323	0.309	0.335	0.418	0.303
UpG									
			H_8	$H_{1'}$	$H_{2'}$	$H_{3'}$			
pG	T_1 (s)		1.7	2.72	1.27	1.21			
	T_1 (s)		(D_8)	3.60	1.74	1.23			
			$\Upsilon_{\mathrm{I}} = 75°$ $P_{\mathrm{syn}} = 0.8$,		$\Upsilon_{\mathrm{II}} = 178°$ $P_{\mathrm{anti}} = 0.2$				
			$\overline{f(\tau_c)} = 4.31 \times 10^{-10}$ s,		$\tau_c = 9.2 \times 10^{-11}$ s				
			j =	1'	2'	3'	4'	5'5"	
	$\langle r_{8j}^{-6}\rangle^{-1/6}$ (nm)	a		0.309	0.245				
		b		0.306	0.265				
Up				H_6	H_5	$H_{1'}$	$H_{3'}$	$H_{5'5''}$	
	T_1 (s)			1.08	2.85	2.62	1.07	0.36	
			$\Upsilon_{\mathrm{I}} = 60°, P_{\mathrm{syn}} = 0.6$,		$\Upsilon_{\mathrm{II}} = 210°, P_{\mathrm{anti}} = 0.4$				
			$\overline{f(\tau_c)} = 4.25 \times 10^{-10}$ s,		$\tau_c = 9.0 \times 10^{-11}$ s				
			j =	5	1'	2'	3'	4'	5'5"
	$\langle r_{6j}^{-6}\rangle^{-1/6}$ (nm)			0.241	0.279	0.261	0.359	0.458	0.339

[a] Calculated from $\Upsilon_{\mathrm{I}}, \Upsilon_{\mathrm{II}}, P_{\mathrm{syn}}, P_{\mathrm{anti}}$.
[b] Deduced from the change in the relaxation rates of 1', 2' after deuteration.

the time in the *anti* orientation. In table 4 are also included the computed mean distances between H^8 and the sugar protons of Guo and between H^6 and the Urd sugar protons. Also included are T_1 values and proton–proton distances after deuteration of H^8. No effect on the relaxation of Urd sugar protons was observed.

The case of UpG at 42°C appears to be slightly more complicated. While Guo appears to behave very similar as in GpU, the Urd moiety shows a small but significant preference for the *syn* conformation (fig. 4a).

The minimum in fig. 4b is rather shallow for both bases, indicating that a shift in both directions of the *syn–anti* equilibrium should be rather easy. Table 4 summarizes these results. The preference for the *syn* conformer is the most striking feature. Otherwise the computed mean proton–proton distances agree reasonably well with those of GpU (table 4). There, is, however, a notable difference: if H^8 of Guo is deuterated the T_1 of the Urd $H_{3'}$ increases by about 15% (from 1.07 s to 1.23 s). This increase, larger than the experimental error (± 5%), is probably significant and reflects an interaction between the Guo base and $H_{3'}$ of Urd. If one corrects for this increase according to eq. (4):

$$\Delta(T_1^{-1})_{H_{3'}} = 1/1.07 - 1/1.23 = 0.121\ \mathrm{s}^{-1}$$

and recomputes the $\Delta f(\tau_c)/\overline{f(\tau_c)}$ as a function of Υ using the corrected $T_1 H^8 = 2.14$ s, instead of 1.7 s, two new minima $\Upsilon = 70°$ and $\Upsilon = 185°$ are found, shifted by about 5° from those obtained before (fig. 4a, —•— line) these minima are also slightly deeper (smaller standard deviation). A recalculation of the *syn -anti* fractions reveals again a better and more pronounced minimum at about 80% *syn* conformer for Guo. The average distance $H^8–H_{3'(U)}$ is then 0.291 nm. If one assumes that this interaction can take place only if Guo is *anti* (as it is evident from molecular models), that is only about 20% of the time, the $H^8–H_{3'(U)}$ distance decreases to 0.222 nm.

4. Discussion and conclusion

The main conclusion of this work is the observation that dinucleoside phosphates have flexible structures in rapid equilibrium between many states. These equilibria include changes between the various glycosidic *syn* and *anti* rotamers, the sugar conformers N and S, the exocyclic rotamers and probably the rotamers around the phosphate group which will affect the stacking of the molecule. From these results we have to conclude that nucleoside diphosphates at room temperature are not good examples for the stacked single stranded helix. As a matter of fact, GpU is probably characteristic of the "end effects" of short helices and no helix persists apparently at room temperature in GpU, a conclusion already reached by optical methods [2,5].

The case of UpG at 42° is a mixed state and about 20% of *anti* conformation of the Guo moiety are probably characteristic of what remains of the stacked *anti–anti* conformation. A quite similar conclusion had been reached from CD melting data on UpG [24]; the amount of stacked UpG at 42° is estimated there to be about 20–25%.

This study clearly indicates that stacking in GpU and UpG is not an important force at ambient temperature. It clarifies also quite a few problems: for example, the definite preference of Guo for the *syn* conformation (figs. 3b and 4b) makes it clear that the stacked *anti–anti* structure contributes only little and any prediction on its nature must be considered with great caution. This implies, that only after consideration of these equilibria, quantitative data can be obtained. It is possible that measurements at low temperature may reveal a larger part of the *anti–anti* stacked form, as had been revealed by a recent low temperature CD study of UpU [25]. Such low temperature relaxation studies are in progress and will be published later.

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