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Nucleoside conformations. 19. Temperature and pH effects on the conformation of guanosine phosphates °

Tran-Dinh Son and Wilhelm Guschlbauer

Service de Biochimie, Departement de Biologie, Centre d'Etudes Nucléaires de Saclay, B.P. N° 2, F-91190 Gif-sur-Yvette, France

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SUMMARY Proton magnetic resonance spectra at 250 MHz were measured as a function of temperature and pH of the three guanosine phosphates. From these data and previously published work the conformational parameters of these compounds were determined. The phosphate group of Guo-5'-P changes its conformation around the C-O bond and its rotation is relatively slow at 20°. At neutral pD the S conformation is favoured and the N form at acid pD. This conformational change is paralleled by a change in exocyclic rotamer distribution and takes place at the pK of the protonation of the base on N^7. Although correlation appears to exist between the various conformations, notable exceptions exist.

INTRODUCTION

Nucleoside and nucleotide conformations have been studied in the last fifteen years by various spectroscopic techniques. Nuclear Overhauser effect studies (NOE) have established that the glycosidic linkage was not rigid, but flexible[1-7], confirming previous ORD and CD measurements[8]. The proportions of the *anti* and *syn* conformations were found to be different for various purine nucleotides; e.g. Ado-5'-P and Guo-5'-P were more *anti* than the corresponding 2' and 3' phosphates, which showed a predominance of the *syn* conformer[3-5].

The flexibility of the ribose moiety in solution has been well established by NMR techniques[9-13]. Altona and Sundaralingam[14,15] have introduced the pseudorotation concept in nucleoside chemistry. They showed that the observed coupling constants can be accounted for by those of two extreme conformers which are in rapid equilibrium: N (3'endo-2'exo) and S (2'endo-3'exo) which are both characterized by a narrow range of phase angles and puckers.

Davies and Danyluk[16] have analyzed all natural ribo- and deoxyribonucleoside-5'phosphates by PMR at 220 MHz using a graphical method based on the pseudorotational concept. Their studies show the predominance of the S conformer in all 5'-phosphates. In a very recent communication[17] these authors have extended this analysis to 3' and 2' phosphates. Again the S conformer

° Part 18 is ref. 32.

appears to be predominant, although the N conformation is exclusively present in the A-form of Nucleic acids and the S conformation only the B-form of DNA[18-20].

It was of interest to investigate by which means the N ⇌ S equilibrium in aqueous solution could be modified and if there were any correlations with the *syn-anti* equilibrium and the rotamer equilibria of the exocyclic group[21].

In the present work we have investigated the 250 MHz PMR spectra of Guo-5'-P and Guo-3'-P and compared them with our previous work on Guo-2'-P (ref. 11). As already observed by NOE[3,5] the pH appears to be the most important external variable to act on the conformation of guanylic acids, while the effect of temperature is rather small.

MATERIAL AND METHODS

Guo-5'-P and Guo-3'-P were purchased from P.L. Laboratories (Milwaukee, Wisc.,USA). Bivalent metallic ions were removed by shaking D_2O solutions of guanylic acids with Chelex 100. After adjusting the pD to neutrality with NaOD or DCl, the samples were lyophilized twice to reduce the concentration of HDO. The pD was taken to be equal to pH + 0.4[22]. No salts or buffers were added. NMR tubes of 5mm diameter were used throughout.

PMR (proton magnetic resonance) spectra were recorded on a CAMECA TSN 250 MHz spectrometer. Chemical shifts were always recorded to better than 0.005 ppm from DSS (2,2-dimethyl-2-silapental-5-sulfonate).

RESULTS AND DISCUSSION

A) Spectral analysis

Figures 1A and 1B show the PMR spectra of the sugar protons of Guo-5'-P at neutral pH at 23° and 70° in aqueous solution. Because of the additional coupling with the phosphate , each of the protons 5' and 5" appears as an octuplet, i.e. a total of 16 peaks for the two protons. While in Ado-5'P under similar experimental conditions[4] the two protons $H_{5'}$ and $H_{5''}$ are nearly equivalent, they are less so in Guo-5'-P. At 70° the peaks become narrower, especially those of $H_{5'}$ and $H_{5''}$, and $H_{4'}$ yields a well-resolved quadruplet, where the phosphate coupling $^4J_{P-H_{4'}}$ of about 1 Hz can be distinguished quite clearly (Figure 1B). Furthermore another long-distance coupling $^5J_{P-H_{3'}}$ (Figure 1B) can be determined which is not observable at room temperature and in general difficult to detect. This difference in the results at 23 and 70° suggests that the CH_2-O-PO_3^{--} group changes its configuration with temperature and that the rotation around the $C_{4'}$-$C_{5'}$ and $C_{5'}O_{5'}$ bonds is relatively slow (on NMR time scale) at room temperature.

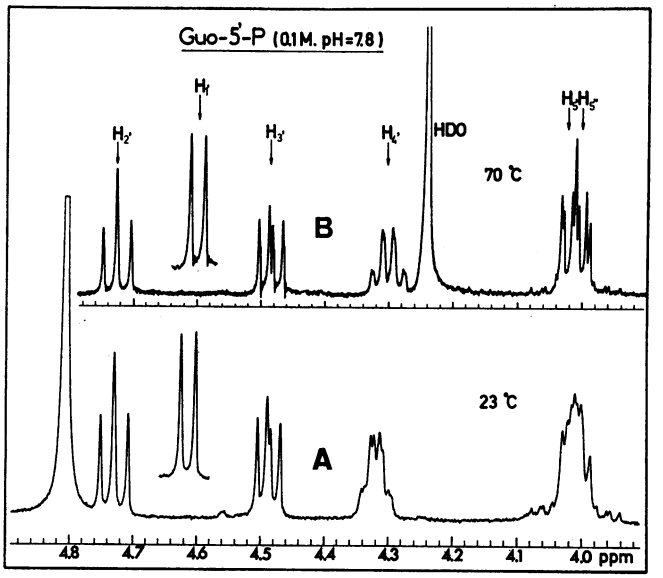

Figure 1: 250 MHz PMR spectrum of Guo-5'-P in D_2O at pH 7.8 recorded at 23°(A) and 70° (B).

It is noteworthy that the form of the $H_{5'}$ and $H_{5''}$ multiplets varies considerably when decreasing the pH (Fig. 2A).

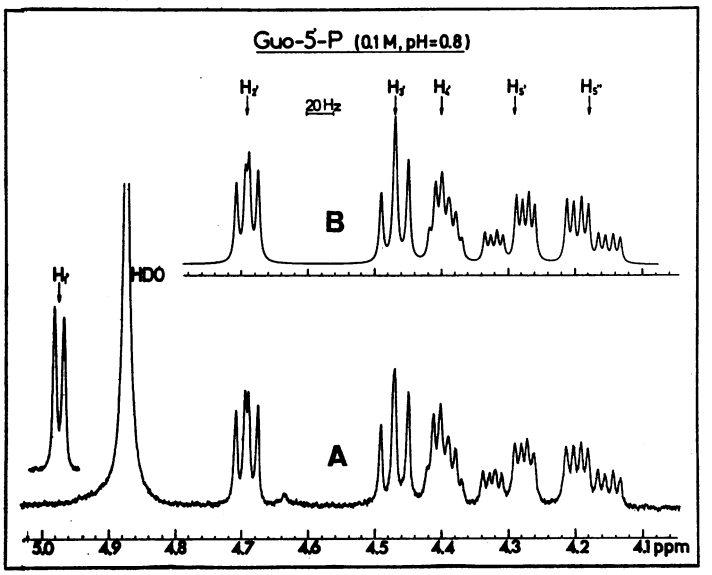

Figure 2: 250 MHz PMR spectrum of Guo-5'-P in D_2O at pH 0.8 at 25° (A) and simulated spectrum (B).

The non-equivalence of the exocyclic protons which partially overlap in neutral medium, is more accentuated at low pD, as seen by the clear separation of the two multiplets. Compared with the spectrum in acid solution (Fig.2A) the protons $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ and $H_{5''}$ are more spread out in neutral medium (Fig. 1A). The protons $H_{5''}$, $H_{5'}$, $H_{4'}$ and $H_{3'}$ form an ABMX system at neutral pD, but an ABCD spectrum in acid solution.

In acid medium the splitting of $H_{1'}$ is decreased; while $H_{2'}$ is a triplet and $H_{3'}$ a quadruplet at neutral pD (Fig. 1A) the inverse is true in acid medium (Fig. 2A). These changes clearly point to profound changes in conformation of the ribose moiety in Guo-5'-P upon acidification of the solution.

Because of the poor separation of the signals of $H_{2'}$ and $H_{3'}$ of the 3'-phosphate even at 250 MHz and their overlap with the HDO peak, its spectrum is less easy to interpret. Heating the solution to about 40° displaces the HDO peak upfield between the protons 3' and 4' (Figure 3A), as had been done with Ado-3'-P[4]. In contrast with Guo-2'-P[11] the two exocyclic protons of Guo-3'-P are virtually equivalent at neutral pD (Fig. 3A), but become well separated at acid pD. Otherwise the acid spectrum of Guo-3'-P shows no dramatic changes and is therefore not shown.

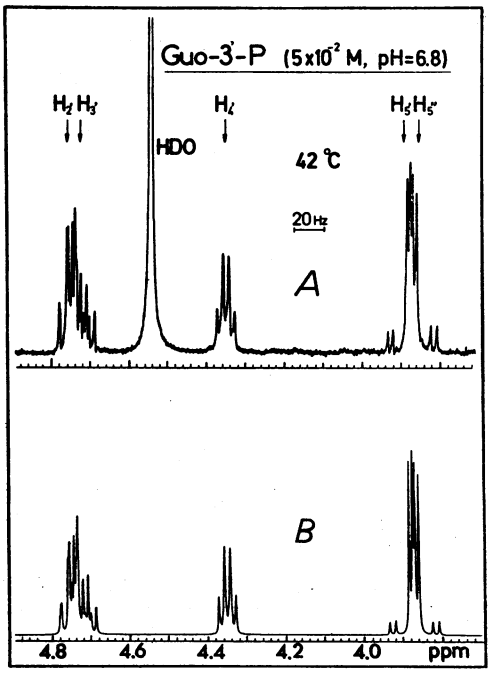

Figure 3: 250 MHz spectrum of Guo-3'-P in D_2O at neutral pH at 42° (A) and simulated spectrum (B).

B) Chemical shifts

a) Effect of temperature. The chemical shifts of the protons of Guo-5'P and Guo-3'-P at neutral pD were followed between 23 and 70° and the changes observed were rather small (Table I). At a given temperature the phosphate displaces the corresponding geminal proton about 0.1 to 0.2 ppm downfield. H^8 of Guo-5'-P is also downfield by 0.17 ppm compared with the 2' and 3' phosphates[3,11]. A specific interaction between the phosphate group and the base is thus indicated[24,25]. Otherwise the sugar protons show only small changes with temperature ($\Delta\delta \leq 0.015$ ppm) except for $H_{4'}$ of Guo-3'-P ($\Delta\delta = 0.036$ ppm). This variation is not related to the ionisation of the phosphate group (see below and Fig. 5). More probable is the proximity of the phosphate at room temperature which will have a greater flexibility at 70° and thus change the electronic environement of $H_{4'}$. A similar reasoning can be applied to the change in chemical shift of H^8 of Guo-5'-P. If one compares the $\Delta\delta_{H^8}$ of Guo-5'-P (0.060 ppm) and Guo-3'-P (0.024 ppm), one notes that the difference is comparable to the change in chemical shift of $H_{4'}$ (0.036 ppm) of Guo-3'-P.

As had been noted above, the movement of the 5'-phosphate group is quite sensitive to temperature changes. The larger *anti* contribution in Guo-5'-P[3,5] makes an interaction of the imidazole part of the base (and thus of H^8) with the phosphate quite probable.

b) pH effects. It has been shown that gel formation of guanylates will only take place in the pH range between the pK of the phosphate and that of the base (pH6.5 to 2.2)[26,27], at low temperature and in the presence of salt. In our conditions (0.05 M nucleotide, absence of salt), gel formation will be negligible even at room temperature.

The spectra of Guo-3'-P were measured at 40°to facilitate the detection of the signals of $H_{2'}$ and $H_{3'}$ (see above). Figures 4 and 5 show the changes of chemical shifts of all non-exchangeable protons of the two nucleotides studied between pH 8 and 0.5. Danyluk and Hruska [25] had already measured the changes of $H_{1'}$ and H^8 of Guo-5'-P at 60 MHz down to pH 3.

Two regions of pH can clearly be distinguished, one corresponding to the pK of the phosphate group around pH 6.6 and the other to that of the base (pK$\sim$ 2.5). In the first region, the protons vicinal to the phosphate carrying carbon are shifted downfield. Only H^8 of Guo-5'-P shows an upfield shift, characteristic of purine-5'-phosphates[4, 24,25], indicating a preponderant *anti* conformation.

In the pH region where the base is titrated, i.e. below pH 4, the H^8 is displaced downfield by about 1 ppm, reflecting the titration on N^7. $H_{1'}$ shows a similar, but much smaller downfield shift ($\Delta\delta\sim 0.18$ ppm), as well

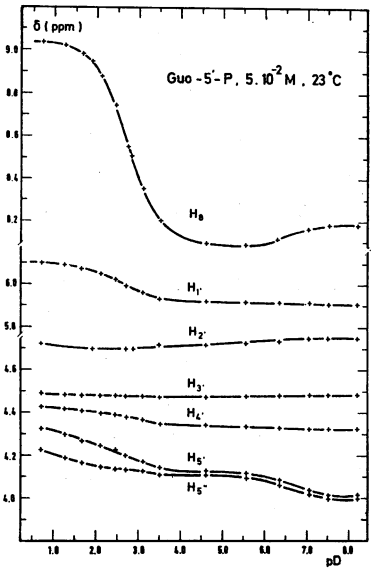

Figure 4: Change in chemical shift of non-exchangeable protons of Guo-5'-P in D_2O as a function of pD.

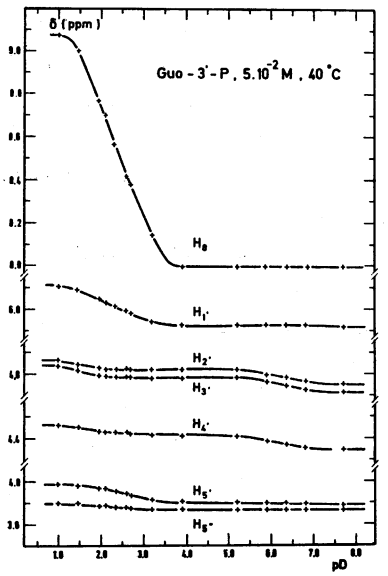

Figure 5: Change in chemical shift of non-exchangeable protons of Guo-3'-P in D_2O as a function of pD.

as all the other sugar protons to a smaller degree. Note the clear separation of the 5'-protons below pH 4, which are nearly equivalent above this pH. Since it is more pronounced for Guo-5'-P (Fig.4), one has an indication of a change in the exocyclic group upon protonation.

Finally, it is noteworthy that below pH 1.5 the 5'-protons of Guo-5'-P continue to change downfield (Fig.4), while they stay constant in Guo-3'-P (Fig. 5). This change is probably a reflection of the second pK of the phosphate group.

C) H-H and H-P coupling constants. Spin-spin coupling constants were determined to better than 0.1 Hz by homo-nuclear decoupling, and/or with the use of program LAOCOON III. They are summarized in Table II. They are in good agreement with the recent data by Davies and Danyluk[16,17]. No significant

TABLE I: Chemical shifts of protons of Guo-5'-P and Guo-3'-P (in ppm from DSS) in D_2O under different experimental conditions

	pD	°C	H^8	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$	$H_{5''}$	$\Delta\delta H_{5'},H_{5''}$
Guo-5'-P	8.2	23	8.179	5.903	4.739	4.496	4.329	4.031	4.008	0.023
0.1 M	8.2	70	8.119	5.906	4.728	4.486	4.304	4.021	4.000	0.021
		Δδ	0.060	0.003	0.011	0.010	0.015	0.010	0.008	
0.05 M	8.4	25	8.172	5.902	4.748	4.484	4.320	4.012	3.992	0.020
	0.9	25	9.030	6.102	4.736	4.490	4.428	4.332	4.228	0.114
Guo-3'-P	8.2	23	8.004	5.918	4.765	4.724	4.369	3.906	3.886	0.020
0.1 M	8.2	70	7.980	5.911	4.748	4.725	4.333	3.890	3.863	0.027
		Δδ	0.024	0.007	0.017	0.001	0.036	0.016	0.023	
0.05 M	7.9	40	7.992	5.916	4.750	4.712	4.344	3.882	3.864	0.018
	1.2	40	9.072	6.104	4.866	4.836	4.460	3.980	3.896	0.084

Table II: Coupling constants J_{HH} and J_{HP} of Guo-5'-P and Guo-3'P in aqueous solution under different experimental conditions.

	pD	°C	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{5'5''}$	$J_{5'P}$	$J_{5''P}$	$^4J_{4'P}$	$^5J_{3'P}$
Guo-5'-P	8.2	23	5.8	5.1	3.8	3.6	3.7	-11.6	4.5	5.1	1.2	<0.3
0.1 M	8.2	70	5.7	5.2	3.9	4.0	4.4	-11.7	5.3	5.7	1.0	0.5
	0.9	23	3.4	4.9	5.5	2.4	2.7	-11.7	4.6	5.5	2.1	<0.4
									$J_{3'P}$		$^4J_{2'P}$	
Guo-3'-P	7.9	40	5.7	5.2	3.4	3.5	3.8	-12.7	7.5		∿0.3	
0.05 M	1.3	43°	4.0	5.2	4.2	2.8	3.8	-12.6	7.0		∿0.3	

temperature effect was observed for the coupling constants of Guo-3'-P and only a rather small temperature effect of the exocyclic group of Guo-5'-P was observed.

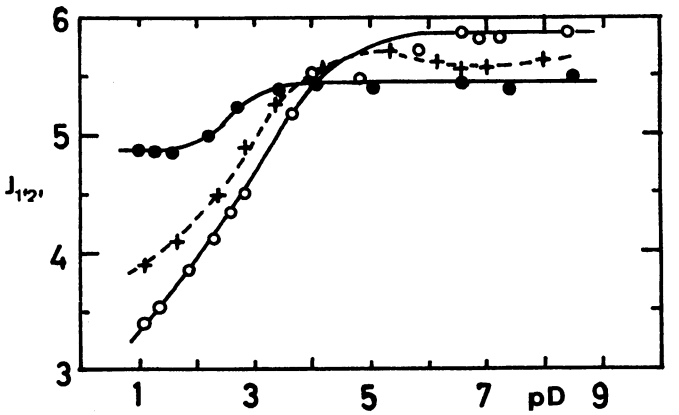

Figure 6: Change in coupling constant $J_{1'2'}$ of the three guanylates as a function of pD. ●-● Guo-2'-P; +-+ Guo-3'-P; o-o Guo-5'-P.

Table III: Pseudorotational parameters of guanylates at different conditions

	A=10.5,B=-1.5,C=0(ref.14.15)'						A=9.3,B=-0.9;C=0 (ref.16,17)					
Conditions	%N	P_N	τ_m^N	%S	P_S	τ_m^S	%N	P_N	τ_m^N	%S	P_S	τ_m^S
Guo-5'-P												
pD 8.2,23°	40	3	41	60	177	41	40	15	41	60	165	41
pD 7.8,70°	41	3	40	59	177	40	41	17	41	59	163	41
pD 1.2,23°	62	-9	42	38	189	42	62	6	41	38	174	41
Guo-3'-P												
pD 8.2,25°	37	-3	40	63	183	40	37	12	40	63	168	40
pD 1.3,43°	50	-20	43	50	200	43	50	-5	39	50	185	39
Guo-2'-P												
pD 7.5,25°	46	6	42	54	174	42	45	21	43	55	159	43
pD 1.2,25°	55	3	45	45	177	45	56	21	47	44	159	47

Errors: %N, %S: ± 0.5 %; P_N, P_S: ± 1.5°; τ_m^N, τ_m^S: ± 0.5°.

For Guo-2'-P the data in ref. 11 were used.

Calculations were performed by a graphical method[33] based on the pseudorotational concept[14,15]: $J_{ij} = x_N \cdot J_{ij}^N + (1-x_n) \cdot J_{ij}^S$; $J_{ij} = A\cos^2\phi_{ij} + B\cos\phi_{ij} + C$

The most striking effect of pD is the decrease of $J_{1'2'}$ and the concominant increase of $J_{3'4'}$, as was already observed for Guo-2'-P[11]. In Figure 6 are summarized the changes of $J_{1'2'}$ as a function of pD for the three guanylates. It should be noted that $J_{1'2'}$ does not change when passing through the phosphate pK, but decreases considerably when the base is titrated. This decrease is largest for Guo-5'-P ($\Delta J_{1'2'}$=2.4) and smallest for Guo-2'-P ($\Delta J_{1'2'}$=0.5 Hz).

D) Ribose conformation. The flexibility of the sugar residue of nucleosides and nucleotides in solution is now firmly established; it is generally assumed to be an equilibrium between two extremes, the conformations N and S, the contributions of which can be determined from coupling constants[14-17].

The results of these calculations using the constants of Altona and Sundaralingam[14,15] and Davies and Danyluk[16,17] are summarized in Table III. While the percentages of N and S conformation are virtually the same in both computations, as well as the pucker τ_m, the phase angle P is about 15° higher, if the constants of Davies and Danyluk are used.

The results in Table III clearly show that the S conformation is favoured in neutral solution, while the N conformation is preferred at acid pD. Furthermore, in accordance with the changes shown in Fig. 6, the increase in N conformation is largest for Guo-5'P and smallest for Guo-2'-P. Still, in Guo-3'P the N and S conformations are only equiprobable in acid medium.

E) Conformation of the exocyclic group.

Table IV shows that the rotamer distribution around the 4'-5'-bond is in favour of the *gauche-gauche* conformation, as it is in most nucleosides and nucleotides. Hruska *et al.*[21], on the basis of an extensive study of nucleosides, had observed that the difference in chemical shifts of the protons $H_{5'}$ and $H_{5''}$ increased with an increase of the *gauche-gauche* rotamer and of the N conformation of the sugar. The cases of Guo-3'-P and Guo-5'-P are excellent illustrations of Hruska's observation, in particular since this phenomenon is verified within a single molecule in two different states of ionisation. When the pD is decreased from 8 to 1, $J_{3'4'}$ increases ($J_{1'2'}$ decreases, Fig.6, Table II) and consequently the contribution of the N conformation increases (Table III), $\Delta\delta_{5'5''}$ and f_{gg}(=P_1) increases (Tables I and IV). $H_{3'}$ is thus in the average more axial at acid pD than in neutral medium. This in turn will reduce the rotational freedom of the exocyclic group and change the environment of $H_{5'}$ and $H_{5''}$; these two latter contributions will evidently increase the separation of the two signals, since the two protons become non-equivalent. The larger downfield shift of $H_{5'}$ and the larger gg contribution suggest that $H_{5'}$ is statistically more close to

$H_{3'}$ than is $H_{5''}$. This leads us to suggest the following scheme:

gauche-gauche *gauche-trans* *trans-gauche*

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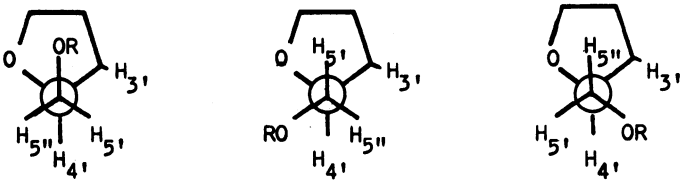

which is opposite to the generally used assignments based on the conclusions of Remin and Shugar[28].

Several other points should also be considered:

1) The temperature can change the rotamer distribution, but will generally little influence the N-S equilibrium (Table III); the temperature effect consists in the acceleration of the rotation of the exocyclic group which will equilibrate the rotamer distribution and the equivalence of protons $H_{5'}$ and $H_{5''}$, but will not very much influence the conformational state

Table IV. Rotamer distribution of the exocyclic group in guanylates under different experimental conditions.

	pD	°C	P_I	P_{II}	P_{III}
Guo-5'-P	8.2	23	0.59	0.21	0.20
	8.2	70	0.46	0.395	0.245
	1.2	23	0.80	0.09	0.11
Guo-3'-P	8.2	40	0.59	0.22	0.19
	1.3	43	0.68	0.22	0.10
Guo-2'-P	7.5	25	0.62	0.25	0.13
	1.2	25	0.62	0.25	0.13

of the sugar moiety. A case, where exocyclic conformation and sugar conformation do not fit, are the 2,2'-anhydro-pyrimidine-nucleosides[29]. An unusual (P=135, 1'exo), virtually rigid conformation and an unfavorable rotamer distribution (f_{gg}=0.18) is found in 2,2'-anhydro-uridine. No temperature effect is observed on the sugar-ring coupling constants, but the

exocyclic group equilibrates at higher temperature, f_{gg} increases to the equilibrium value and simultaneously $\Delta\delta_{5'5''}$ decreases towards zero.

2) That the solvent may have a considerable effect on the rotamer distribution is also exemplified by anhydrouridine and its derivatives[29]. The *gauche-gauche* conformation is favoured in D_2O, but unfavoured in DMSO. Again $\Delta\delta_{5'5''}$ decreases with an increase of f_{gg}. We conclude that $\Delta\delta_{5'5''}$ will increase with temperature only if f_{gg} is greater than 0.33 at room temperature; if $f_{gg}<0.33$, heating will decrease $\Delta\delta_{5'5''}$.

3) The influence of the *anti-syn* equilibrium on the in-equivalence of the 5'-protons does not seem to be conclusive. NOE studies[3] had shown that Guo-5'-P was more *anti* than Guo-2'-P and Guo-3'-P. $\Delta\delta_{5'5''}$ of Guo-3'-P and Guo-5'-P are quite similar and lower than for Guo-2'-P. No change in the rotamer distribution (Table IV) is observed in the case of Guo-2'-P upon acidification, while there is a very large effect upon Guo-5'-P, and a lesser, but unequal effect upon Guo-3'-P (only $J_{4'5'}$ decreases, Table III).

F) Rotation around the $C_{5'}$-$O_{5'}$-bond. Tsuboi *et al.*[30] had studied the conformational equilibria of alcoylphosphates in aqueous solution and have concluded that the following coupling constants describe the *trans* and *gauche* conformations between H and P

$^3J_{t'}$ = 28 Hz and $^3J_{g'}$ = 1.5 Hz.

Blackburn *et al.*[31] and Davies and Danyluk[16] have proposed the relation

$$^3J_{HCOP} = 16.3 \cos^2\phi_{HP} - 4.6 \cos\phi_{HP}$$

which will yield $J_{g'}$ = 1.8 Hz and $J_{t'}$ = 20.9 Hz, in fair agreement with the constants above.

In the case of Guo-5'-P, $J_{P-5'}$ and $J_{P-5''}$ are quite low which indicates a predominance of the g'g' conformer, independent of the $J_{t'}$ value used. A slight increase in these coupling constants at elevated temperature points to the decrease of the g'g' contribution. The long-range couplings $^4J_{P-4'}$ and $^5J_{P-3'}$ change from 1.2 to 1.0, and from less than 0.3 to 0.5, respectively. A parallel narrowing of the proton resonances coupled with the phosphate with temperature, indicates that the rotation of the phosphate around the $C_{5'}$-$O_{5'}$ bond must be rather slow at room temperature.

G) Conformation around the glycosidic bond.

Although no measurements have been made in this work which could give information about the *syn-anti* equilibrium, we can compare the present data with the NOE measurements reported previously. It had been shown that Guo-5'-P was more *anti* at neutral pH than the two other guanylates[3]. A similar situation was observed with adenylates[4,5]. Upon acidification, Guo-3'-P and Guo-2'-P assumed preferentially the *syn* conformation.

In a recent relaxation and NOE study on several nucleosides of the purine series in liquid ammonia[6,7], it had been suggested that a correlation existed between nucleoside conformation and glycosidic bond conformation: the N form with the *anti* conformation, the S form with the *syn* conformation. Similar correlations seem to exist in the crystal structures of nucleosides studied[14].

Our present data and previous NOE measurements[3] summarized in Table V lead, however, to opposite conclusions, i.e. the acid mediated increase of the *syn* conformation is paralleled by an increase in the N conformation. From Table V it is, however, also clear that there is no direct relationship between these phenomena. As a matter of fact, Guo-5'-P which shows the largest change in sugar conformation upon titration, shows no increase of the *syn* conformation. Guo-3'-P which is quite strongly *syn* in neutral medium and 78 percent *syn* at low pH has still equal amounts of N and S forms. Guo-2'-P which shows the largest increase in *syn* conformation shows an intermediate change in sugar conformation.

Table V: Summary of conformational parameters of guanylates in different environements in aqueous solution.

	pD	°C	$f_{gg} = P_I$	$K_N = \frac{[N]}{[S]}$	$K_s = \frac{[syn]}{[anti]}$
Guo-5'-P	7	25	0.59	0.67	1.1
	7	70	0.46	0.69	
	1	25	0.80	1.65	1.0
Guo-3'-P	7	25	0.59	0.60	2.6
	1	25	0.68	1.00	3.5
Guo-2'-P	7	25	0.62	0.85	1.7
	1	25	0.62	1.20	4.0

CONCLUSIONS

The conformational parameters of guanylates summarized in Table V show quite clearly that there is no straightforward explanation for the acid-mediated conformational changes observed. Qualitatively, an increase in the gg rotamer and of the *syn* conformation appears to be associated with a larger contribution of the N form, but notable exceptions exist. The correlation *gauche-gauche* - N agrees - qualitatively - with the observations of Hruska *et al.* [21], the *syn* - N correlations is contrary to the conclu-

sions of Lüdemann *et al.*[6,7]. We wish to insist, however, that these correlations appear to be rather qualitative and the exceptions are frequent.

After acceptation of this manuscript, a paper by Lee, Evans and Sharma (J. Biol. Chem.250 (1975) 1290-1296) on the conformation of Ado-5'-P and Guo-5'-P and their 8-aza-analogues came to our attention. These authors show that the aza-analogues have a greater *syn* contribution than the natural nucleotides. This is paralleled by an increase of the N form of sugar puckering in agreement with our data, but a decrease of the *gauche-gauche* rotamer contribution, in contrast with our results on acid induced conformational changes. The results of Lee *et al.* on Guo-5'-P in neutral solution are identical to those presented here.

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