Conformation of Thymidine 3′,5′-Cyclic Phosphate in Aqueous Solution. Compatibility of the Information from the Lanthanide-Ion Probe and the Spin-Coupling Constants

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The Pr(III)- and Eu(III)-induced shifts of ^{1}H nuclear magnetic resonances of thymidine 3′,5′-cyclic phosphate were observed in D_2O solution. The results indicated that the induced shifts were primarily of dipolar origin and that the magnetic susceptibility tensor had effective axial symmetry. The ratios of shifts intrinsic to the lanthanide-substrate 1:1 complex were determined after correcting for the contributions of the complex formation shifts (diamagnetic terms). The Gd(III)-induced perturbations of the spin-lattice relaxation times were also observed, and the ratios of relaxation rates were obtained. A computer search was then performed to find out the molecular conformation of this cyclic nucleotide which best fitted the shift and relaxation ratios. As a result the phosphate-ring part was found to have a typical chair conformation. The effective symmetry axis of the magnetic susceptibility tensor coincided with the bisector of the OPO angle almost completely. It was rather difficult, however, to establish the conformation of the deoxyribose ring from the shift and relaxation ratios alone. The use of the additional information from the vicinal ^{1}H—^{1}H coupling constants ($J_{1'2'}$ and $J_{1'2''}$) was necessary for obtaining a reasonable conformation of the entire molecule. The compatibility of the information from the lanthanide-ion probe and the spin coupling constants ($^3J_{HH}$ other than $J_{1'2'}$ and $J_{1'2''}$ and $^3J_{HP}$) is discussed along with the limitations of both methods.

The chemical shift and relaxation perturbations induced by paramagnetic lanthanide cations[1-6] have been increasingly utilized for the determination of conformations of biologically important molecules.[7,8] The advantage of this lanthanide-ion probe method lies in its potentiality of yielding information on relative arrangements of nuclei located within a relatively long distance (approximately 10 Å) and in various directions from the bound lanthanide cation. The information obtainable by this method is particularly valuable when the molecule under study has a portion for which neither vicinal nor long-range spin-coupling data are available. For the phosphodiester linkage in dinucleoside monophosphates no spin coupling is present and accordingly, no information is obtainable as to the torsional angles about the P–O bonds. It seems therefore interesting to apply the lanthanide-ion probe method to conformation studies of this class of molecules. However, such flexible molecules should be treated with caution. For 5′-nucleotides the results obtained by this method[9-11] are not quite consistent with the conclusions derived from the spin-coupling constants.[12-14] Therefore, it is important to check the compatibility of these two methods with an appropriate molecule related to dinucleoside monophosphates. For such a purpose it has been found that thymidine 3′,5′-cyclic phosphate (3′,5′-cTMP) is particularly suited on the following grounds. (a) The binding of the lanthanide cation to 3′,5′-cTMP and to a dinucleoside monophosphate would be similar, since either has a phosphodiester linkage as the binding site. (b) Unlike other 3′,5′-cyclic nucleotides, the analysis of ^{1}H and ^{13}C NMR spectra of 3′,5′-cTMP has given an exceptionally complete set of the ^{1}H–^{1}H, ^{1}H–^{31}P, and ^{13}C–^{31}P coupling constants.[15,16] From the analysis of these coupling constants it has been concluded that the deoxyribose and cyclic phosphate rings of 3′,5′-cTMP are locked in a rigid conformation. Consequently, the result from the lanthanide-ion probe may be compared easily with that from the spin-coupling constants.

Experimental

Materials and Preparation of Sample Solutions. Thymidine 3′,5′-cyclic phosphate (3′,5′-cTMP) was purchased from Sigma Chemical Co. and was used without further purification. The nitrates of Eu(III), Pr(III), Gd(III), and La(III) (purity better than 99.9%) obtained from Nakarai Chemical Co. were used for measuring the induced shifts or the relaxation-time perturbations.

The shifts induced by Pr(III) and the "complex formation" shifts induced by La(III)[17,18] were observed in D_2O solutions by the successive dilution method.[19,20] The concentrations of 3′,5′-cTMP in the initial solutions were 0.307 and 0.253 M for the measurements with Pr(III) and La(III), respectively. The molar ratio of a lanthanide ion to the substrate was kept constant [1.186 for Pr(III) and 1.185 for La(III)] throughout a series of dilution measurements. The shifts induced by Eu(III) were also observed but unfortunately the resonance peaks due to $H_{5'}$ and $H_{5''}$ were overlapped with the HDO peak as a result of the shift and the peaks arising from $H_{2'}$ and $H_{2''}$ could not be separated clearly. The "pH" (uncorrected pH meter reading) was adjusted to about 2.0. Precipitation occurred at higher pH. A Radiometer PHM 26 pH meter was used with a long thin Ingold combination electrode (Catalog No. 6030—04). For the measurements of Gd(III)-perturbed proton relaxation times, a small amount of Gd(III) nitrate solution was added successively to the 0.19 M solution of 3′,5′-cTMP. Thus the concentration of Gd(III) was varied from 20 to 140 μM in steps of 20 μM.

NMR Measurements. The NMR spectra were obtained with a Hitachi R-22/FT spectrometer operating at 90 MHz for ^{1}H. The probe temperature was kept at 37 °C. The chemical shifts were determined by a frequency counter using a frequency-sweep mode. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as an internal standard. Spin-lattice relaxation times (T_1) were obtained by means of a standard 180°—τ—90° pulse sequence.

Data Analysis. Computer programs were developed to derive a conformation which best fitted not only the chemical

shift and relaxation perturbations induced by lanthanide ions but also the conformation-dependent coupling constants. The main features of the programs are described in the Appendix. Computations were carried out with a HITAC 8800/8700 system at the Computer Centre of the University of Tokyo.

Results and Discussion

Lanthanide-Induced Shifts. The induced chemical shifts of all the protons of 3',5'-cTMP could be observed only with Pr(III), whereas the results with Eu(III), Nd(III), and Yb(III) were incomplete because of unfavorable line overlappings and/or line broadenings. The Pr(III)-induced shifts observed by the successive dilution method were corrected for the complex formation shifts which were determined using diamagnetic La(III). Next the ratios of the Pr(III)-induced shifts of protons $H_{5'}$, $H_{5''}$, $H_{4'}$, $H_{2'}$, $H_{2''}$, $H_{1'}$, H_6, and H_{Methyl} relative to that of H_3, were calculated and extrapolated to zero substrate concentration [as for the numbering of protons, see Fig. 1(a)]. Such a procedure was previously shown to be appropriate for obtaining experimentally the shift ratios intrinsic to the 1 : 1 complex between the substrate molecule and the lanthanide ion.[19,20] The shift ratios thus obtained with Pr(III) are listed in Table 1, together with the results with Eu(III). Although the Eu(III)-induced shifts were observed only for $H_{3'}$, $H_{5'}$, $H_{4'}$, $H_{1'}$, H_6, and H_{Methyl} the shift ratios derived from them were not much different from the corresponding ones obtained with Pr(III). This means that the induced shifts for these protons are mainly of dipolar origin and that the susceptibility tensor has effective axial symmetry. Therefore, the shift ratios for the above six protons could be used for conformation studies with the McConnel-Robertson equation.[21] Since only the Pr(III)-induced shifts were obtained for $H_{5''}$, $H_{2''}$, and $H_{2'}$, the shift ratios for these three protons were used in the conformation search with a weight factor of 1/10 relative to the other protons.

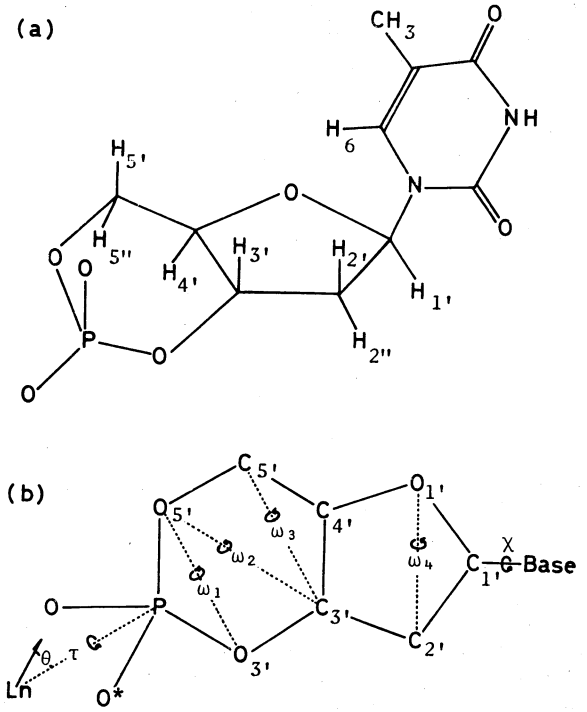

Fig. 1. Schematic representation of the structure of thymidine 3',5'-cyclic phosphate. (a), Numbering of the protons; (b), ring-puckering and torsional coordinates.

TABLE 1. THE OBSERVED AND CALCULATED SHIFT AND RELAXATION RATIOS FOR THYMIDINE 3',5'-CYCLIC PHOSPHATE

^1H[a]	Shift Ratio			
	Obsd		Calcd[b]	
	Pr(III)	Eu(III)	A	B
3'	1.000	1.000	1.000	1.000
5'	0.798	0.831	0.825	0.813
4'	0.623	0.642	0.634	0.643
5''	0.581[c]	—	0.556	0.569
2''	0.322[c]	—	0.350	0.369
2'	0.235[c]	—	0.249	0.252
1'	0.167	0.206	0.206	0.251
6	0.073	0.075	0.096	0.107
CH_3	−0.060	−0.018	−0.068[d]	−0.028[d]

^1H[a]	Relaxation Ratio		
	Obsd	Calcd[b]	
		A	B
3'	1.000	1.000	1.000
5'	0.561	0.555	0.557
4'	0.186	0.152	0.133
5''	0.161	0.169	0.164
2''	0.101	0.078	0.082
2'	0.101	0.106	0.101
1'	0.080	0.028	0.028
6	0.091	0.083	0.073
CH_3	0.046	0.030[d]	0.015[d]

a) As for the notations of the protons, see Fig. 1(a). b) The values in the columns A and B were obtained, respectively, from Conformations A and B of Table 2. c) The weights for these values were 1/10 of those for the other data. d) An average position of the methyl protons was assumed to be the center of the triangle formed by them.

Gd(III)-Induced Relaxation Rate Enhancement. The relaxation rates for the nuclei of a Gd(III)-bound molecule are to a good approximation proportional to $\langle r_i^{-6} \rangle$, where r_i refers to the distance between Gd(III) and the ith nucleus.[7,9] In the present study the change of the spin-lattice relaxation time (T_1) with successive addition of a small amount of Gd(III) was followed. The observed spin-lattice relaxation rates ($1/T_1^{obsd}$) are plotted against the concentrations of Gd(III) in Fig. 2. As shown in a previous paper,[22] the slope of each plot is proportional to the relaxation rate of each nucleus in the Gd(III)-bound molecule. Using the data thus obtained, the relaxation ratios were calculated as listed in Table 1, where the relaxation rate for $H_{3'}$ was chosen as a reference.

Spin-Coupling Constants. The ^1H–^1H and ^1H–^{31}P coupling constants of 3',5'-cTMP at pD 7.2 were obtained by Blackburn *et al.*[15] No appreciable varia-

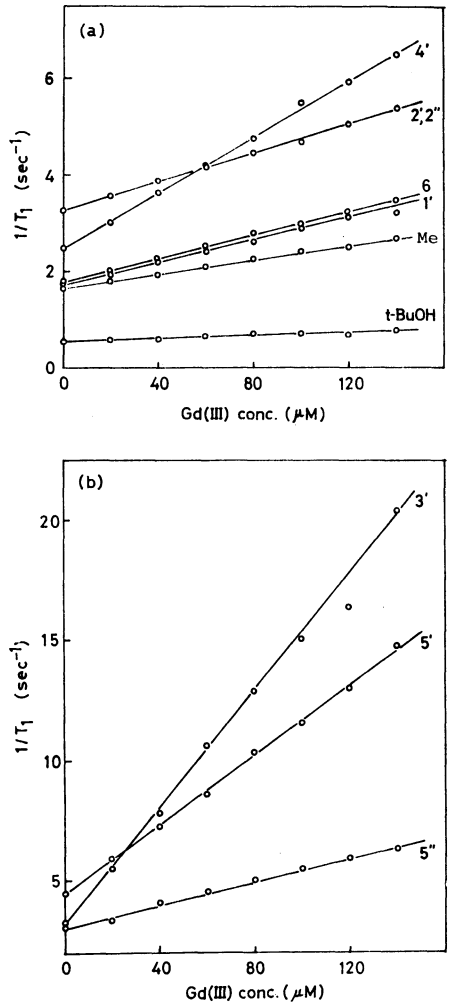

Fig. 2. Dependence of the spin-lattice relaxation rate ($1/T_1^{obsd}$) on the concentration of Gd(III). (a), $H_{1'}$, $H_{2'}$, $H_{2''}$, $H_{4'}$, $H_{6'}$, and H_{Methyl}; (b), $H_{3'}$, $H_{5'}$, and $H_{5''}$. The concentration of 3',5'-cTMP was 0.19 M. *t*-BuOH (*ca.* 20 mM) was used as an internal standard.

tions of the spin-coupling constants from these values were found with the addition of approximately equimolar La(III) at pD 2.0. This indicated that the conformation of 3',5'-cTMP remained unchanged upon complex formation with the lanthanide cation. It is also known that for 3',5'-cTMP no protonation occurs between pH 2.0 and 7.2. Therefore, the spin-coupling constants given by Blackburn *et al.* will be used in the following discussion.

Conformation Search. A search for the conformation of 3',5'-cTMP which best fitted all the observed data was carried out in the following way. A conformation based on the structure of uridine 3',5'-cyclic phosphate in a crystal was assumed at first. This model was employed because no crystallographic data were available for 3',5'-cTMP itself. By modifying this initial model a conformation was obtained which gave the best fit between the observed and calculated values of the shift and relaxation ratios. Finally a further adjustment of molecular parameters was performed by taking a few key spin-coupling constants into consideration. Such a process and the results obtained will be described below in more detail.

From the crystal data of uridine 3',5'-cyclic phosphate (molecule A)[23] all the necessary parameters, *viz.*, bond lengths, bond angles, ring-puckering angles, and the torsional angle around the glycosidic bond, were obtained and transferred to 3',5'-cTMP. The four ring-puckering angles $\omega_1-\omega_4$ [shown in Fig. 1(b)] as well as the torsional angle around the glycosidic bond were chosen as variable parameters[24] in the iteration process of fitting the calculated shift and relaxation ratios to the observed values. These are defined as the dihedral angles between the two planes, each of which is indicated below by the three atoms in a parenthesis: ω_1 ($PO_{5'}O_{3'}$) and ($O_{5'}O_{3'}C_{3'}$); ω_2 ($O_{3'}O_{5'}C_{3'}$) and ($O_{5'}C_{3'}C_{5'}$); ω_3 ($O_{5'}C_{3'}C_{5'}$) and ($C_{2'}C_{5'}C_{4'}$); ω_4 ($C_{4'}C_{2'}$-$O_{1'}$) and ($C_{2'}O_{1'}C_{1'}$). Since each dihedral angle is equivalent to the torsional angle around the imaginary bond XY in the atomic sequence A–X–Y–B as given in Table 2, its sign was given according to the IUPAC-IUB conventions on the definition of a torsional angle.[25] The torsional angle χ around the glycosidic bond is defined with respect to the sequence $O_{1'}$–$C_{1'}$–N_1–C_6. To describe the conformation of a five-membered ring two puckering angles are generally needed. However, only one (ω_4) was sufficient for the deoxyribose ring of 3',5'-cTMP because of the interlocking of this and the phosphate rings. In Table 2 the initial values of $\omega_1-\omega_4$ and χ are given as Conformation I.

Table 2. Molecular parameters of thymidine 3',5'-cyclic phosphate

Molecular Parameter[a]	Conformation		
	I	A	B
ω_1(P–$O_{5'}$–$O_{3'}$–$C_{3'}$)	−135°	−116°	−114°
ω_2($O_{3'}$–$O_{5'}$–$C_{3'}$–$C_{5'}$)	−179°	−189°	−189°
ω_3($O_{5'}$–$C_{3'}$–$C_{5'}$–$C_{4'}$)	−121°	−121°	−126°
ω_4($C_{4'}$–$C_{2'}$–$O_{1'}$–$C_{1'}$)	−160°	−187°	−152°
χ($O_{1'}$–$C_{1'}$–N_1–C_6)	77°	135°	84°
θ		4°	4°
τ		−101°	−66°

a) As for the definitions of ω, χ, θ, and τ, see the text and Fig. 1(b).

The shift and relaxation ratios were calculated on the assumption that the lanthanide cation was located on the bisector of the OPO angle. This assumption was substantiated by previous studies,[9-11,24,26] particularly for the early members of the series of lanthanide cations like Pr(III) and Eu(III).[27] The direction of the symmetry axis of susceptibility tensor was made variable. Two parameters θ and τ were used to specify this direction, the former being the angle between the symmetry axis and the OPO bisector and the latter the dihedral angle between the plane involving the symmetry axis and the P atom and the plane defined by Ln, P, and O* [see Fig. 1(b)]. The angle τ could be regarded as the torsional angle around the Ln–P linkage in the sequence formed by a point on the symmetry axis–Ln–P–O* and, therefore, its sign was given in the same way as for ω's and χ. The distance between Ln(III) and P was fixed to 3.0 Å (corresponding

to the Ln–O distance of 2.6 Å) which is in the range generally accepted.

The agreement factor (R_1)[28] defined below is a useful measure for evaluating the degree of fit between the observed and the calculated values.

$$R_1 = \left\{ \frac{\sum_i v_i (p_i^{\text{obsd}} - p_i^{\text{calcd}})^2 + \sum_i w_i (q_i^{\text{obsd}} - q_i^{\text{calcd}})^2}{\sum_i v_i (p_i^{\text{obsd}})^2 + \sum_i w_i (q_i^{\text{obsd}})^2} \right\}^{1/2} \quad (1)$$

Here p_i and q_i refer to the shift ratio and the relaxation ratio for the ith nucleus, respectively, and v_i and w_i are the corresponding weights (both set to unity unless otherwise mentioned). After minimizing the R_1 value by varying ω_1–ω_4, χ, θ, and τ, Conformation A of Table 2 was obtained as the best-fit model. The shift and relaxation ratios calculated for Conformation A are listed in Table 1 as Calcd A. The R_1 value for Calcd A was 0.048 [using the shift ratios observed by Pr(III)], which is comparable with the value (0.037) obtained for hydroxy-L-proline.[22] The R_1 value was found rather insensitive to ω_4 since it is related only to $H_{1'}$, H_6, and H_{Methyl} which have small shift and relaxation ratios. Therefore, the two spin-coupling constants $J_{1'2'}$ and $J_{1'2''}$ were included in the conformational search in order to add further information for determining ω_4. The Karplus-type equation derived by Altona and Sundaralingam[29] was used for this purpose. The agreement factor (R_2) defined below was used in this search.

$$R_2 = \left\{ \frac{\left(\sum_i v_i (p_i^{\text{obsd}} - p_i^{\text{calcd}})^2 + \sum_i w_i (q_i^{\text{obsd}} - q_i^{\text{calcd}})^2 \right) + \sum_k u_k (J_k^{\text{obsd}} - J_k^{\text{calcd}})^2}{\sum_i v_i (p_i^{\text{obsd}})^2 + \sum_i w_i (q_i^{\text{obsd}})^2 + \sum_k u_k (J_k^{\text{obsd}})^2} \right\}^{1/2} \quad (2)$$

In this equation p_i, q_i, v_i, and w_i are the same as in Eq. (1), and J_k and u_k refer to the coupling constant and the corresponding weight, respectively. The ratio of u_k to v_i (except those for $H_{5''}$, $H_{2''}$, and $H_{2'}$ as indicated in Table 1) or w_i was set to 0.004 somewhat arbitrarily. With this value, shift ratios, relaxation ratios, and coupling constants contributed almost equally to the structure determination. The resultant best-fit conformation is indicated as Conformation B in Table 2, and the shift and relaxation ratios calculated for this model are listed in the column Calcd B of Table 1. The R_1 value (*not* R_2) for Calcd B was 0.070. A stereoscopic drawing of Conformation B is shown in Fig. 3.

The following points may be noted from the results described above. (1) The conformation of the phosphate ring could be determined by the lanthanide-ion probe method. There is no significant difference between phosphate-ring conformation determined here (Conformations A and B) and that of 3′,5′-cUMP in a crystal. Both may be called a chair form (see Fig. 3). (2) The small value of θ as determined for either Conformation A or B indicates the virtual coincidence of the symmetry axis of susceptibility tensor with the bisector of the OPO angle. Such a result ensures the validity of assuming this coincidence in the studies of other molecules having a phosphodiester bond. (3) It was difficult to obtain definite information on the positions of $H_{1'}$, H_6, and H_{Methyl} by the lanthanide-ion probe method alone. This is mainly because the distances between the lanthanide cation and these protons are relatively long (8.1, 7.0, and 9.0 Å for $H_{1'}$, H_6, and H_{Methyl}, respectively). (4) A reasonable conformation of the entire molecule could be obtained by the use of $J_{1'2'}$ and $J_{1'2''}$ in addition to the shift and relaxation data. The conformation of the deoxyribose ring may be described as 4′-*exo* (Fig. 3) in agreement with the conclusion of Blackburn *et al.*[15] However, it should be remembered that the parameters in the Karplus-type equation used here were based on the data of ribosylnucleosides and ribosylnucleotides.[29] If a different set of parameters is found to hold for the deoxyribose ring of 3′,5′-cyclic phosphates, the deoxyribose part of Conformation B should be modified accordingly. The conformation of the phosphate and deoxyribose rings determined here is roughly similar to the corresponding one of adenosine 3′,5′-cyclic phosphate reported by Barry *et al.*[24] According to these authors, the distance between Ln and $H_{4'}$ calculated from the relaxation data was not consistent with the structure determined from the shift ratios. Such discrepancy was not encountered in our case, since both the shift and relaxation ratios were used simultaneously. (5) In Conformation B the torsional angle around the glycosidic bond χ is close to that of molecule A of 3′,5′-cUMP in a crystal[23] and is in the anti range.[30] The value of χ in Conformation A seems less reliable. (6) If R_1 has a shallow minimum with respect to a variable parameter (*e.g.*, ω_4), a minor decrease in R_1 does not necessarily reflect a closer approach to the true conformation. Therefore, such

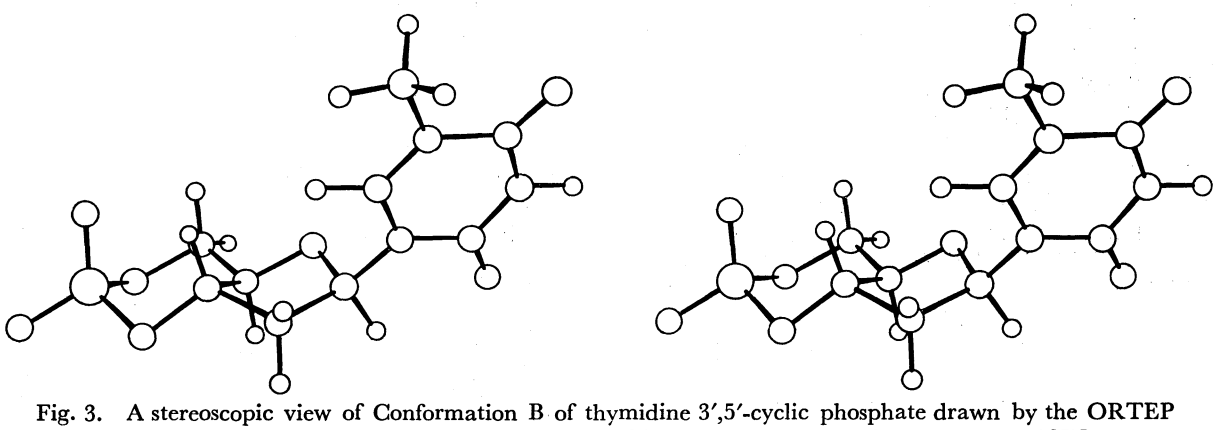

Fig. 3. A stereoscopic view of Conformation B of thymidine 3′,5′-cyclic phosphate drawn by the ORTEP program [C. K. Johnson, ORTEP. Report ORNL-3794, Oak Ridge National Laboratory (1965)].

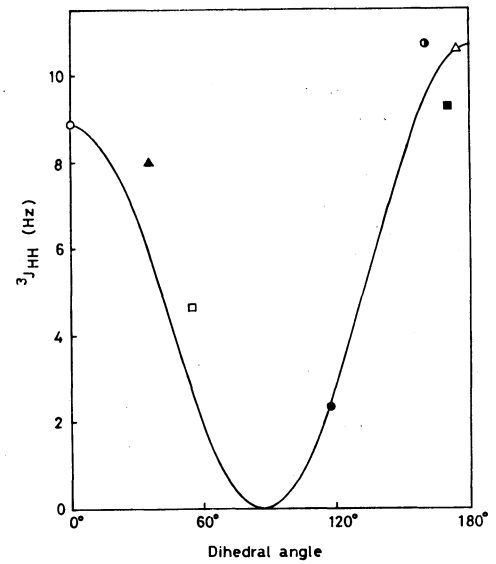

Fig. 4. The vicinal ^1H—^1H coupling constants $^3J_{HH}$ of thymidine 3′,5′-cyclic phosphate plotted against the corresponding H–C–C–H dihedral angles in Conformation B. ○, $J_{1'2''}$; ▲, $J_{2'3'}$; □, $J_{4'5'}$; ●, $J_{1'2'}$; ◑, $J_{2''3'}$; ■, $J_{3'4'}$; △, $J_{4'5''}$. The solid curve indicates the variation of $^3J_{HH}$ with the dihedral angle as expressed by the Karplus-type equation with the coefficients of Altona and Sundaralingam.[29]

a case should be treated with caution.

In Fig. 4 the vicinal ^1H–^1H coupling constants ($^3J_{HH}$) are plotted against the H–C–C–H dihedral angles in Conformation B. Although the observed $^3J_{HH}$'s qualitatively follow the trend expressed by the Karplus-type equation (solid curve), there are considerable deviations of $J_{2'3'}$ and $J_{4'5'}$ from the curve. It is likely that these deviations reflect the limitations of the semi-empirical Karplus-type equation rather than the errors in the dihedral angles of Conformation B. Any possible torsional angle about the $C_{2'}$–$C_{3'}$ bond cannot give good fits of both $J_{2'3'}$ and $J_{2''3'}$ with the curve in Fig. 4. Blackburn *et al.* also noted using another Karplus-type expression that the observed $^3J_{HH}$ values were not in satisfactory agreement with the values calculated for a 4′-*exo* conformation, though it gave better fits than the other conformations.

Blackburn *et al.* determined three vicinal ^1H–^{31}P coupling constants ($^3J_{HP}$), namely $J_{3'P}$, $J_{5'P}$, and $J_{5''P}$, to be 1.7, 2.2, and 20.4 Hz, respectively.[15] The corresponding H–C–O–P dihedral angles (ϕ_{HP}) in Conformation B are 46°, 63°, and 179°, respectively. Such a relationship between $^3J_{HP}$ and ϕ_{HP} is in agreement with the results obtained earlier by Tsuboi and coworkers,[31–33] White and Verkade,[34] and Hall and Malcolm[35] from the spectral studies of some molecules containing the H–C–O–PO_2^- system. This gives another support to the use of the $^3J_{HP}$–ϕ_{HP} relationship to structural studies of dinucleoside monophosphates.[36]

Conclusion

The lanthanide-ion probe method is useful for determining the conformation of the phosphate ring of thymidine 3′,5′-cyclic phosphate. The resultant phosphate ring conformation is compatible with what is expected from the vicinal ^1H–^1H and ^1H–^{31}P coupling constant. The direction of the symmetry axis of magnetic susceptibility tensor virtually coincides with the bisector of the OPO angle. If two ^1H–^1H coupling constants $J_{1'2'}$ and $J_{1'2''}$ are used in the conformation search to supplement the shift and relaxation data, a reasonable conformation of the entire molecule (Conformation B depicted in Fig. 3) is obtained.

Appendix

Conformation search based on the shift and relaxation ratios requires a considerable amount of computation when the molecule under study has many degrees of internal freedom, *e.g.*, the internal rotation around a single bond and the puckering of a ring. Although the outlines of computer programs developed by other workers have been published,[6,9,28] it would be still worth while to describe the main procedure of our program which we believe has some unique aspects.

Our program consists of two parts, namely, PCS-I and PCS-II. PCS-I is used to compute internal coordinates from atomic coordinates when the latter data are available from a crystallographic analysis. The bond length, bond angle, internal-rotation (torsional) angle, and ring-puckering angle are adopted as internal coordinates. As described in the text, the latter two angles are defined in accord with the IUPAC-IUB conventions. The internal coordinates thus obtained are used as an initial set of parameters for the conformation search by PCS-II. If no crystal data are available, an initial set of internal coordinates is assumed appropriately by referring to the data of analogous molecules.

PCS-II has the functions of computing (1) the space-fixed Cartesian coordinates of atoms from the internal coordinates (the reversal of the process carried out by PCS-I), (2) the shift and relaxation ratios using the McConnel-Robertson equation and the first term in the Solomon-Bloembergen equation, respectively, and (3) the agreement factor (R). It is also possible to calculate the spin-coupling constants from the Karplus equation (or its modification) and to include the spin-coupling terms in the agreement factor. PSC-II is also used to minimize the R value by varying the internal coordinates. This is performed in the following manner.

(a) According to user's instructions the internal coordinates are divided into two groups, variable and invariable. The former usually includes the position of the lanthanide ion and the direction of the principal axis of magnetic susceptibility tensor in addition to internal-rotation and ring-puckering angles. Bond lengths are almost always invariable (fixed to the initial values), and bond angles are varied only in some special cases.

(b) The R values are computed for the conformation with the initial set of variable parameters (ϕ_1^0, ϕ_2^0, ···, and ϕ_n^0) and the $2n$ conformations with the new sets ($\phi_1^0 \pm h_1$, ϕ_2^0, ···, and ϕ_n^0), ;(ϕ_1^0, $\phi_2^0 \pm h_2$, ···, and ϕ_n^0), ···, and (ϕ_1^0, ϕ_2^0, ···, and $\phi_n^0 \pm h_n$). Here, h_1, h_2, ···, and h_n are the variable increments which should be set appropriately by the user. If the smallest one among the R values for the new conformations is smaller than that for the initial conformation, the initial set is replaced by the set corresponding to the smallest. The same process is repeated until no further variations of internal coordinates reduce the R value. In order to gain fast convergence it is advantageous to use large h_i values in the first

search and to make h_i values smaller and smaller in the subsequent searches. This can be performed either automatically or stepwise at user's option.

Recently we extended PCS-II to treat the flexible molecules which exist in two or more conformers with various populations. The application of such a function of PCS-II will be reported separately.

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