

Advanced Nuclear Magnetic Resonance Lanthanide Probe Analyses of Short-Range Conformational Interrelations Controlling Ribonucleic Acid Structures†

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ABSTRACT: An advanced method was developed for lanthanide-probe analyses of the conformations of flexible biomolecules such as nucleotides. The new method is to determine structure parameters (such as internal-rotation angles) and population parameters for local conformational equilibria of flexible sites, together with standard deviations of these parameters. As the prominent advantage of this method, the interrelations among local conformations of flexible sites may be quantitatively elucidated from the experimental data of lanthanide-induced shifts and relaxations and vicinal coupling constants. As a structural unit of ribonucleic acids, the molecular conformations and conformational equilibria of uridine

3'-monophosphate in aqueous solution were analyzed. The stable local conformers about the C3'-O3' bond are the G^+ ($\phi' = 281 \pm 11^\circ$) and G^- ($\phi' = 211 \pm 8^\circ$) forms. The internal rotation about the C3'-O3' bond and the ribose-ring puckering are interrelated; $97 \pm 5\%$ of the C3'-endo ribose ring is associated with the G^- form while $70 \pm 22\%$ of the C2'-endo ribose ring is associated with the G^+ form. An interdependency also exists between the internal rotation about the C4'-C5' bond and the ribose-ring puckering. These short-range conformational interrelations are probably important in controlling the dynamic aspects of ribonucleic acid structures.

The conformational properties of nucleotide units have been found to be related with the biological functions of tRNAs. For example, as shown by the conformation analyses of dinucleoside monophosphates (Watts & Tinoco, 1978), hypermodified nucleosides at the 3' side of the anticodon maintain the flexibility of the anticodon loops for regulated efficiency of the translation process. 2-Thiouridine derivatives contribute to the correct codon recognition (Yokoyama et al., 1979a) and to the thermostability (Watanabe et al., 1979) through the conformational characteristics that the C3'-endo form is much more stable than the C2'-endo form. However, according to the conformation obtained by X-ray analysis, hypermodified nucleoside Q appears to be important in the process other than protein synthesis (Yokoyama et al., 1979b). The conformational properties of nucleotide units are also important for DNA. In the left-handed Z-DNA form of d-(CpGpCpGpCpG), guanosine residues take an unusual *gt*-C3'-endo-syn form rather than the *gg*-C2'-endo-anti form as found in the B-DNA form (Wang et al., 1979). This polymorphism of DNA may be related with the biological functions of DNA. For understanding nucleic acid functions, some detailed analyses of the dynamic conformational properties of nucleic acids are indispensable.

For the conformation analyses of nucleotides in aqueous solution, nuclear magnetic resonance (NMR) spectroscopy is useful. Chemical shifts, spin-coupling constants, nuclear relaxation rates, and nuclear Overhauser effect have been used for elucidating local conformations (Davies, 1978a). However, these data are not quite sufficient for studying overall conformations of nucleotides. For such studies, the lanthanide-probe analyses have been made for a number of flexible molecules (Barry et al., 1974; Birdsall et al., 1975; Geraldès & Williams, 1978, 1979; Dobson et al., 1978; Geraldès, 1979). Previously Inagaki et al. (1978) developed a useful method

of lanthanide-probe analysis by a nonlinear least-squares search and elucidated the structures and fractional populations of conformers of uridine 5'-monophosphate (pU).¹ However, this method was not applicable to more complicated molecules with many flexible sites. Accordingly, in the present study, we have developed a new computer program (COFLEM) on the basis of highly improved algorithm. The overall conformer mixture is now treated as a combination of local conformational equilibria for flexible sites. This advanced lanthanide-probe analysis with the program COFLEM is applied first for studying the conformational equilibrium of the ribose 3'-phosphate moiety of uridine 3'-monophosphate (Up),¹ a basic nucleotide unit of polyribonucleotide chains.

The conformational properties about the C3'-O3' bond of nucleotides have not been elucidated in detail, because only three vicinal coupling constants, $J(H3',P)$, $J(C2',P)$, and $J(C4',P)$, are available (Lee et al., 1976; Ezra et al., 1977; Smith et al., 1973; Alderfer & Ts'o, 1977). The interrelation between the ribose-ring puckering and the internal rotation about the C3'-O3' bond (Ezra et al., 1977; Alderfer & Ts'o, 1977) has not been quantitatively estimated by analyses of vicinal coupling constants alone. In the present lanthanide-probe analysis of Up, however, the internal-rotation angles of the G^+ and G^- forms about the C3'-O3' bond were determined. The interrelations between the ribose-ring puckering and the internal rotation about the C3'-O3' and C4'-C5' bonds were also elucidated quantitatively. These findings provide an experimental basis for the interpretation of dynamic structure features of RNA in solution and the biological roles of naturally occurring 2-thiopyrimidine nucleosides in tRNAs.

Experimental Procedures

Materials. Uridine 3'-phosphate (Up) was purchased from Sigma and was used without further purification. The nitrates of Pr(III), Eu(III), and Gd(III) of more than 99.9% purity were obtained from Nakarai Chemical Co., and the lanthanum

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¹ Abbreviations used: Up, uridine 3'-monophosphate; pU, uridine 5'-monophosphate.

oxide of more than 99.99% purity was purchased also from Nakarai Chemical Co. and dissolved in nitric acid (or hydrochloric acid) to obtain lanthanum nitrate (or lanthanum chloride).

Sample Solutions. The pH of sample solutions (direct pH meter reading) was measured by the use of a Radiometer PHM26 pH meter with a long thin combination electrode. For the measurements of ^{13}C spin-lattice relaxation rates, the $^2\text{H}_2\text{O}$ solution of Up (140 mM, pH 4.0) was treated with chelating agents to eliminate residual paramagnetic metal ions. For the measurements of the lanthanide-induced shift ratios of Up in $^2\text{H}_2\text{O}$ solution (pH 1.8), the successive dilution method (Wittstruck, 1972; Inagaki et al., 1975a,b) was employed. The initial concentrations of Up and lanthanide ion (praseodymium or europium) were both 80 mM for ^1H shift measurements but were 400 and 800 mM, respectively, for ^{13}C shift measurements. Such initial solutions were diluted successively with $^2\text{H}_2\text{O}$ (pH 1.8) so that the molar ratio of lanthanide ion to Up was kept constant throughout a series of induced shift measurements. For the measurements of Gd(III)-perturbed nuclear relaxation rates of Up in $^2\text{H}_2\text{O}$ solution, the concentration of Gd(III) ion was varied from 0.01 to 1 mM. For the best separation of ^1H resonances of Up and for avoiding the formation of Up-Gd(III) 2:1 complex, Eu(III) (23 mM) and La(III) (200 mM) were added to the $^2\text{H}_2\text{O}$ solution of Up (200 mM). Similarly for ^{13}C relaxation measurements, Pr(III) (80 mM) and La(III) (100 mM) were added to the $^2\text{H}_2\text{O}$ solution of Up (350 mM).

NMR Measurements. NMR spectra were measured with a Bruker WH270 spectrometer operating at 270 MHz for ^1H and at 67.9 MHz for ^{13}C and with a Hitachi R-22/FT spectrometer operating at 22.6 MHz for ^{13}C . The probe temperature was kept at 30 °C. Spin-lattice relaxation rates ($1/T_1$) were obtained by a standard $180^\circ\text{-}\tau\text{-}90^\circ$ pulse sequence. As the internal standard for the Gd(III)-perturbed bulk relaxation as well as for chemical shifts, *tert*-butyl alcohol and dioxane were used for ^1H and ^{13}C nuclei, respectively.

Data Analyses. A new computer program, COFLEM (Conformations of FLExible Molecules), was written in the present study (to be described in a later section) and was operated on the HITAC 8800/8700 system at the Computer Center of the University of Tokyo.

Experimental Results

Effects of Binding with Lanthanide Ion. It is the conformation of the complex of monoanionic Up molecule and a Ln(III) ion that can be analyzed by the lanthanide-probe method. Therefore, it is necessary to examine if the conformation of monoanionic Up molecule is affected by the binding with lanthanide ion. Accordingly vicinal spin-coupling constants were measured of free monoanionic Up molecule [$^2\text{H}_2\text{O}$ solution of Up (140 mM), pH 4.0] and of monoanionic Up molecule complexed with La(III) ion [$^2\text{H}_2\text{O}$ solution of Up (140 mM) and LaCl_3 (700 mM), pH 1.5]. In the former system, all the Up molecules ($\text{p}K_a = 1.0$) are monoanionic. In the latter system, the fractional population of free un-ionized Up molecules is as low as 2%, and 93% of monoanionic Up molecules are complexed with La(III) ion, as estimated with the association constant ($K = 23 \text{ M}^{-1}$) for the similar molecule of pU (Inagaki et al., 1978). As shown in Table I, the vicinal spin coupling constants for pairs of ribose protons are only slightly different (at most 0.8 Hz) between the free and complexed monoanionic Up molecules. These indicate that the fractional populations of the C2'-endo/C3'-endo forms of the ribose ring and the *gg* form about the exocyclic bond are not affected more than a few percent by the binding with

Table I: Spin Coupling Constants (Hz) of Up

| | obsd | | calcd | | |
|----------|------------------|------------------|-------|-----|-----|
| | free | complexed | I | II | III |
| H1',H2' | 4.9 | 5.3 | 5.6 | 6.7 | 5.8 |
| H2',H3' | 5.2 | 5.3 | 5.3 | 5.3 | 5.1 |
| H3',H4' | 5.2 | 4.4 | 5.3 | 4.2 | 5.0 |
| H4',H5' | 2.9 | 3.1 | 3.5 | 3.9 | 3.2 |
| H4',H5'' | 4.2 | 4.2 | 4.3 | 4.4 | 4.2 |
| H3',P | 8.2 | 8.6 | 2.7 | 9.8 | 8.2 |
| C2',P | 4.2 ^a | 4.2 ^a | 2.7 | 3.8 | 3.8 |
| C4',P | 4.6 ^a | 4.3 ^a | 8.8 | 4.3 | 4.6 |

^a These are accurate within 0.6 Hz while others are accurate within 0.2 Hz.

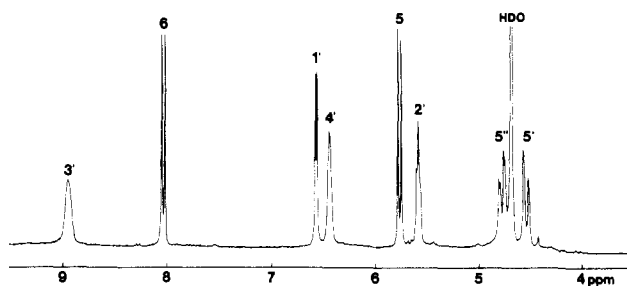


FIGURE 1: 270-MHz ^1H NMR spectrum of Up (80 mM) with $\text{Pr}(\text{NO}_3)_3$ (80 mM) in $^2\text{H}_2\text{O}$ solution at pH 1.8 and 30 °C.

lanthanide ion. Furthermore, the vicinal spin-coupling constants for the phosphorus nucleus (P) and ribose nuclei (H3', C2', and C4') are practically the same for the free and complexed Up molecules. These observations clearly indicate that the conformation of Up molecule in the Ln(III)-Up complex is primarily the same as that of free monoanionic Up molecule. Therefore, the lanthanide-probe method is suitable for elucidating the conformational properties of monoanionic Up molecules in aqueous solution.

The vicinal spin coupling constants (J) as listed in Table I were used for studying the conformation equilibrium of Up in aqueous solution. In the computer analyses, vicinal spin coupling constants were calculated with generalized Karplus equations, namely eq 1 and 2 (Davies, 1978a; Lee et al., 1976)

$$J(^1\text{H}, ^1\text{H}) = 10.2 \cos^2 \alpha - 0.8 \cos \alpha \quad (1)$$

$$J(^1\text{H}, ^1\text{H}) = 10.5 \cos^2 \alpha - 1.2 \cos \alpha \quad (2)$$

$$J(^1\text{H}, ^{31}\text{P}) = 18.1 \cos^2 \alpha - 4.8 \cos \alpha \quad (3)$$

$$J(^{13}\text{C}, ^{31}\text{P}) = 9.5 \cos^2 \alpha - 0.6 \cos \alpha \quad (4)$$

for the ribose ring and exocyclic bond, respectively, and eq 3 and 4 (Lee & Sarma, 1976; Govil & Smith, 1973) for $J(^1\text{H}, ^{31}\text{P})$ and $J(^{13}\text{C}, ^{31}\text{P})$, respectively.

Ln(III)-Induced Shifts. The shifts of ^1H and ^{13}C resonances induced by Pr(III) or Eu(III) were corrected for the complex formation shifts as estimated by the use of diamagnetic La(III) (Bleaney et al., 1972). After such corrections, the ratios of the Ln(III)-induced shifts were extrapolated to zero Up concentration to obtain the shift ratios for the Up-Ln(III) 1:1 complex [successive dilution method (Inagaki et al., 1975a,b)]. The shift ratios of ^1H resonances relative to H4' and the shift ratios of ^{13}C resonances relative to C4' are listed in Table II. The ^1H shift ratios obtained with Eu(III) and with Pr(III) are practically equal to each other, indicating that the induced shifts are of pseudocontact origin and the magnetic susceptibility tensor effectively has the axial symmetry. Accordingly, the Pr(III)-induced shift ratios of ^1H and ^{13}C resonances (Figure 1) were used for studying the conformation equilibrium

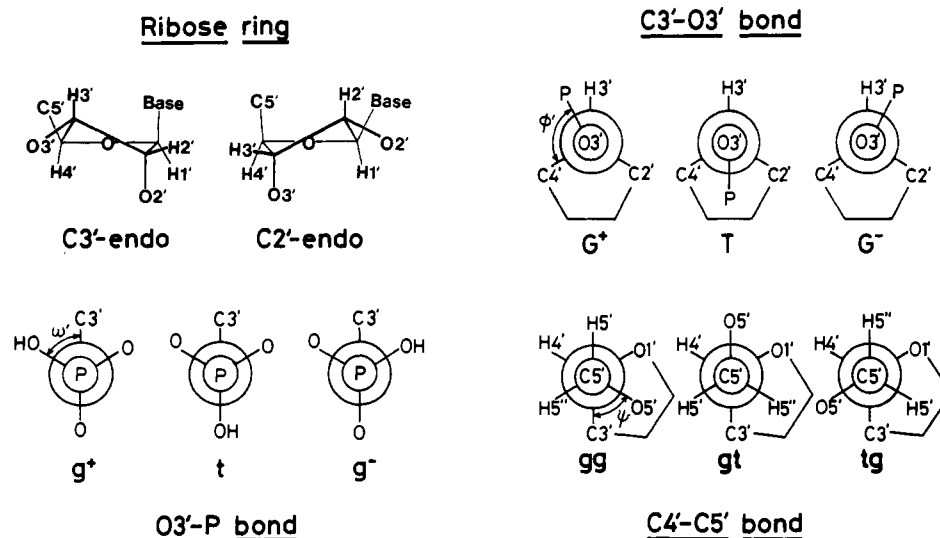


FIGURE 2: Local conformations of ribose 3'-phosphate moiety.

Table II: Shift and Relaxation Ratios of Up

| | shift ratio | | | | | relaxation ratio | | | |
|------|-------------|-------|-------|------|------|------------------|------|-------|------|
| | obsd | | calcd | | | obsd | | calcd | |
| | Eu | Pr | I | II | III | Gd | I | II | III |
| H1' | 0.33 | 0.33 | 0.77 | 0.56 | 0.48 | 0.38 | 0.64 | 0.46 | 0.24 |
| H2' | 0.54 | 0.56 | 1.16 | 0.68 | 0.64 | 0.72 | 0.63 | 0.61 | 0.68 |
| H3' | 2.04 | 1.91 | 1.81 | 1.79 | 1.77 | 2.12 | 1.53 | 2.02 | 2.13 |
| H4' | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| H5' | 0.30 | 0.36 | 0.46 | 0.49 | 0.33 | 0.31 | 0.45 | 0.57 | 0.38 |
| H5'' | 0.40 | 0.51 | 0.61 | 0.61 | 0.49 | 0.53 | 0.76 | 0.94 | 0.60 |
| H5 | -0.03 | 0.00 | b | b | b | 0.22 | b | b | b |
| H6 | 0.12 | 0.15 | b | b | b | 0.18 | b | b | b |
| C1' | | 0.43 | 0.77 | 0.59 | 0.56 | 0.58 | 0.62 | 0.51 | 0.40 |
| C2' | | 0.69 | 1.33 | 0.94 | 0.92 | 1.13 | 1.28 | 1.15 | 0.99 |
| C3' | | 2.02 | 2.00 | 1.88 | 1.92 | 2.07 | 2.44 | 2.49 | 2.31 |
| C4' | | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| C5' | | 0.40 | 0.58 | 0.62 | 0.54 | 0.91 | 0.68 | 0.79 | 0.66 |
| C2 | | 0.04 | b | b | b | a | b | b | b |
| C4 | | 0.05 | b | b | b | a | b | b | b |
| C5 | | -0.04 | b | b | b | 0.28 | b | b | b |
| C6 | | 0.03 | b | b | b | 0.25 | b | b | b |

^a Not measured. ^b Not included in calculations.

of Up. In the computer analyses, the shift ratios of *i*th nuclei relative to the *s*th nucleus were calculated by eq 5, where $\Delta\nu_i$

$$\frac{\Delta\nu_i}{\Delta\nu_s} = \frac{\langle [3 \cos^2 \theta_i - 1] / r_i^3 \rangle}{\langle [3 \cos^2 \theta_s - 1] / r_s^3 \rangle} \quad (5)$$

is the induced shift of the *i*th nucleus and r_i and θ_i are the spherical polar coordinates of the *i*th nucleus in the coordinate system of the principal magnetic axes with Ln(III) as the origin (Barry et al., 1974).

Gd(III)-Induced Relaxation Rates. The spin-lattice relaxation rates of Up induced by Gd(III) were corrected for the bulk relaxation rates as estimated by the use of internal standard (*tert*-butyl alcohol and dioxane do not form specific complexes with Gd(III) ion). After such corrections, the ratios of the Gd(III)-induced relaxation rates of ¹H and ¹³C resonances (Table II) were used for studying the conformation equilibrium of Up. In the computer analyses, the ratio of the relaxation rates ($1/T_{1M}$) of the *i*th and *s*th nuclei was calculated with eq 6. This equation applies to the case where the

$$(1/T_{1M})_i / (1/T_{1M})_s = \langle r_i^{-6} \rangle / \langle r_s^{-6} \rangle \quad (6)$$

dipolar interactions between the electron spin of Gd(III) ion and the nuclear spins (*i*th and *s*th) are characterized by a

common correlation time (τ_c). Accordingly, for proton-bearing ¹³C nuclei of Up (free from lanthanide ion), the spin-lattice relaxation rates were measured, and, by the use of the bond length of C-H = 0.109 nm, the correlation times were found to be practically the same (C1' 54, C2' and C3' 56, C4' 58, C5' 56, C5 55, and C6 58 ps). Therefore the correlation times of the ¹³C nuclei of Up appear to be primarily determined by overall rotation of the molecule. This suggests in turn that the correlation times for protons are also controlled by the same mechanism. Accordingly, in the present computer analysis, eq 6 was used for calculating relaxation ratios of ¹H as well as ¹³C nuclei of Up in aqueous solution.

Computer Program COFLEM

Conformational Averaging. Nucleoside 3'-monophosphate molecules are known to be flexible in aqueous solution; the ribose phosphate moiety has several flexible sites, including the puckering of ribose ring (C2'-endo and C3'-endo) and the internal rotation about the P-O3' (ω'), O3'-C3' (ϕ'), C4'-C5' (ψ) bonds (Figure 2). Because of such flexibility, the molecular conformation is to be expressed with an ensemble of a number of conformers in aqueous solution. The interconversion of those conformers is fast enough relative to the NMR time scale so that the observed Ln(III)-induced shifts, Gd(III)-induced relaxation rates, and vicinal spin coupling constants are the averages of the values for individual conformers as weighted with the fractional populations.

Conformation Tree. For the four flexible sites of nucleoside 3'-monophosphate, the potential-energy profile will have a number of minima. A simplifying assumption is made in the present analysis that an internal coordinate represents the accessible region of one potential-energy minimum. The four flexible sites (Figure 2) are assigned to the four levels of branching from the root to the top of the "conformation tree" (Figure 3). For the *n*th flexible site with N_n local conformations, a branch at the (*n* - 1)th level forks out to N_n branches at the *n*th level. These branches need to be specified with N_n values of relevant internal coordinates and N_n values of fractional populations. However, since the sum of this local set of fractional populations is equal to unity, the number of independent fractional populations is $N_n - 1$.

Characterization of Conformers. For a molecule with *M* flexible sites, there are a total of $N_1 N_2 \cdots N_M$ overall conformers. The structure of a conformer is described by a set of internal coordinates along the line from the root to the top of the conformation tree. The fractional population of a

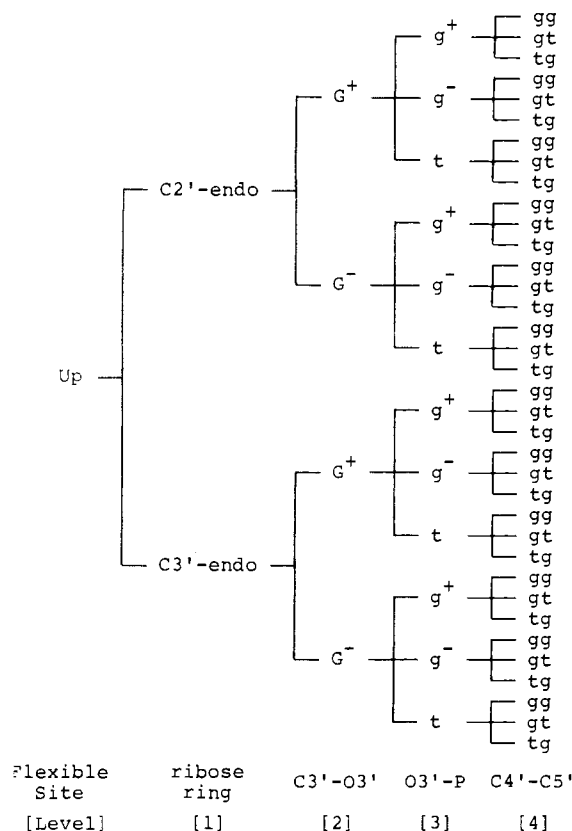


FIGURE 3: Conformation tree for ribose 3'-phosphate moiety.

conformer is given by the product of the fractional populations of local conformations. The total sum of the fractional populations of conformers is equal to unity because of the normalization condition at each level of branching. Local conformation properties (internal coordinate and fractional population) of a flexible site (n th level) are possibly interrelated with those of an adjacent site (lower m th level, as is the case for the puckering of the ribose ring and the internal rotation about the C3'-O3' bond). Otherwise, the local conformational properties of a flexible site (n th) on different branches may be taken as common, regardless of the branching at the lower m th level. Thus, the ensemble of conformers is specified by the conformation tree.

Agreement Factor. Conformation search is now to find the conformation tree that satisfactorily reproduces the observed values of lanthanide-induced shifts and relaxation rates, vicinal coupling constant, and nuclear Overhauser effects. The structure parameters (internal coordinates and others) and population parameters (fractional populations of local conformations) are adjusted so that the calculated values agree best with the observed values. The quality of the fit is judged by the agreement factor R , defined as

$$R = [\sum_j \omega_j (X_j^{\text{obsd}} - X_j^{\text{calcd}})^2] / [\sum_j \omega_j (X_j^{\text{obsd}})^2] \quad (7)$$

Here ω_j is the weight for the j th observable value X_j .

Structure and Population Parameters. In the first step of conformation search, the initial values of the structure and population parameters are set up. The structure parameters include the coordination parameters of lanthanide ion and the orientation parameters for the principal magnetic axis as well as the internal coordinates. Structure parameters are divided into two groups, variable and invariable. The former usually includes internal-rotation angles. Bond lengths are almost always invariable (fixed to the initial values), and bond angles are varied only in some special cases. The population pa-

rameters in the initial set include independent fractional populations of local conformation, without regard to possible interrelations among the fractional populations at different levels of the conformation tree.

Minimization of R Factor. The agreement factor R is computed first for the initial structure parameters (S_1^0, S_2^0, \dots) and population parameters (F_1^0, F_2^0, \dots). The R value is then calculated for a new set of population parameters ($F_1^0 \pm \Delta F_1, F_2^0, \dots$) but with the same structure parameters. If the R factor is decreased with $F_1^0 \pm \Delta F_1$, F_1^0 is replaced by $F_1^0 \pm \Delta F_1$; otherwise not. This process is performed successively on all the other population parameters, F_2, F_3 , etc. The variable increment ΔF may be made stepwise smaller. This cycle is repeated until the R factor is minimized, and thus a refined set of population parameters is obtained. Such refinements of population parameters are included as a subroutine in the adjustments of structure parameters. Thus, for a new set of structure parameters ($S_1^0 \pm \Delta S_1, S_2^0, \dots$), the R factor is minimized by the refinements of population parameters. Only if the minimized R factor is smaller than that with the initial set of parameters is S_1^0 replaced by $S_1^0 \pm \Delta S_1$. This procedure is followed successively on all the other structure parameters, S_2, S_3 , etc. The variable increments may be made stepwise smaller. The whole cycle is repeated until no further variations of parameters reduce the R factor. It should be noted that, in such minimization treatment with many parameters, there are possibly a number of false sub-minima together with a real minimum. Therefore it is important to start from a number of initial sets of the structure and population parameters and to examine if the same minimum is reached.

Standard Deviations. After the "real" minimum is reached, the most probable values for variable parameters may be calculated by the method of least squares. The elements of the Jacobian matrix for shift ratios, relaxation ratios, spin coupling constants, and nuclear Overhauser effect are calculated numerically. After iterations of successive approximation processes with the method of least squares, the most probable values and standard deviations are obtained for variable parameters. Such standard deviations are useful for examining the reliability of the most probable values of structure and population parameters.

Refinement of Model. In the first stage of conformation search, common structure and population parameters are used for a flexible site (common branching is used). Actually, however, because of possible steric interactions, electrostatic interactions, and others, the structure and population parameters of a flexible site may depend on the local conformations at another flexible site in the proximity. Such interrelation between different flexible sites is taken into account in the second stage of conformation search by incorporating an additional number of appropriate parameters. This new set of structure and population parameters is adjusted so as to minimize the R factor further, and the standard deviations of variable parameters are calculated. If the introduction of the new set of parameters corresponds to the valid physical model, the R factor is appreciably reduced. The standard deviations of variable parameters, in particular of the parameters of interrelating flexible sites, will be remarkably decreased. Such a refinement process is followed for each of the appropriate combination of flexible sites, until no further process improves the R factor and standard deviations of variable structure and population parameters. Thus, the conformation tree (with the most probable structure and population parameters) is obtained that best reproduces the observed values of lanthanide-induced

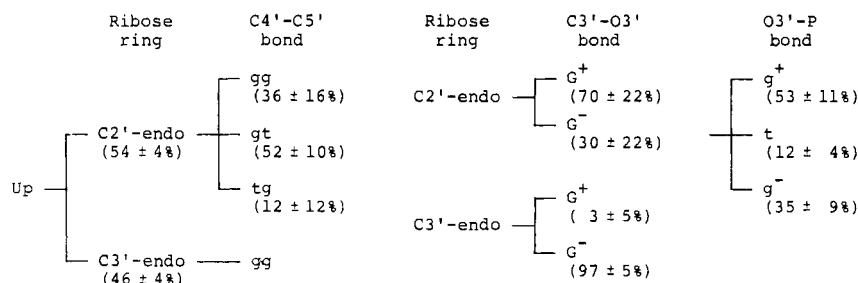


FIGURE 4: Fractional populations of local conformations of Up [solution III, $\phi' = 281 \pm 11^\circ$ (G^+) and $211 \pm 8^\circ$ (G^-)].

shifts and relaxation rates, spin coupling constants, and nuclear Overhauser effects.

Conformation Search

The conformational equilibrium of the ribose phosphate moiety of the Up molecule was analyzed by the use of a total of 26 observed data, namely, 8 vicinal spin-coupling constants (Table I), 9 lanthanide-induced shift ratios, and 9 gadolinium-induced relaxation ratios (Table II).

Invariable Parameters. In the conformation search, Ln(III) ion was set on the bisector of monoanionic OPO angle, 3 nm away from the phosphorus atom as in the cases of thymidine 3',5'-phosphate (Inagaki et al., 1975a) and pU (Inagaki et al., 1978). The structures of the two puckering forms (C3'-endo and C2'-endo) of the ribose ring were taken from X-ray crystal data (Yathindra & Sundaralingam, 1975). As shown in Figure 2, three forms were taken up both for the O3'-P bond [g^+ ($\omega' = 60^\circ$), t ($\omega' = 180^\circ$), and g^- ($\omega' = 300^\circ$)] and for the C4'-C5' bond [gg ($\psi = 60^\circ$), gt ($\psi = 180^\circ$), and tg ($\psi = 300^\circ$)].

Solution I. As for the internal rotation about the C3'-O3' bond, the three-rotamer model with conventional internal-rotation angles [G^+ ($\phi' = 300^\circ$), T ($\phi' = 60^\circ$), and G^- ($\phi' = 180^\circ$)] (see Figure 2) was tested first. The interrelations among the conformational properties (population parameters) of the four flexible sites were not taken into account. The fractional populations about the four flexible sites were adjusted as follows: ribose ring (C3'-endo $48 \pm 8\%$, C2'-endo $52 \pm 8\%$), C3'-O3' bond (G^+ $17 \pm 5\%$, T 0% , G^- $83 \pm 5\%$), O3'-P bond (g^+ $23 \pm 15\%$, t 0% , g^- $77 \pm 15\%$), and C4'-C5' bond (gg $62 \pm 19\%$, gt $25 \pm 13\%$, tg $13 \pm 15\%$). Thus, the fractional population of the T form about the C3'-O3' bond was reduced to zero. However, in solution I, the calculated values of the vicinal coupling constants J (H3',P) and J (C4',P) (Table I), shift ratios of H2' and C2', and relaxation ratios of H1' and H3' (Table II) significantly deviate from the observed values, and thus the R factor is still as large as 0.28. Accordingly, certain refinement of the conformer model system is required.

Solution II. In the second stage of refinement, the internal-rotation angles (ϕ') about the C3'-O3' bond were taken up in the set of structure parameters. However, the fractional population of the T form again was reduced to zero. Accordingly, in subsequent refinements, this T form about the C3'-O3' bond was discarded. The internal-rotation angles (ϕ') of the G^+ and G^- forms as well as fractional populations were adjusted as follows: ϕ' (G^+ $275 \pm 34^\circ$, G^- $215 \pm 28^\circ$), ribose ring (C3'-endo $37 \pm 5\%$, C2'-endo $63 \pm 5\%$), C3'-O3' bond (G^+ $38 \pm 53\%$, G^- $62 \pm 53\%$), O3'-P bond (g^+ $61 \pm 11\%$, t $4 \pm 3\%$, g^- $35 \pm 10\%$), and C4'-C5' bond (gg $57 \pm 11\%$, gt $25 \pm 8\%$, tg $18 \pm 9\%$). The calculated values of the coupling constants J (H3',P) and J (C4',P) (Table I), shift ratios of H2' and C2', and relaxation ratios of H1' and H3' (Table II) now agree much better with the observed values than those in the

case of solution I, and in fact, the R factor is reduced to 0.16 for solution II. However, the standard deviations of the ϕ' angles and of the fractional populations about the C3'-O3' bond are appreciably large.

Solution III. In the third stage of refinement, therefore, the interrelations between the ring puckering of ribose ring and the internal rotation about the two exocyclic bonds were taken into account. Thus, two independent sets of fractional populations about the exocyclic bonds (G^+ , G^- , gg , gt , and tg) were included as variable parameters (one set for the C3'-endo form and the other set for the C2'-endo form of the ribose ring). Then, the nine independent population parameters and two structure parameters (ϕ') were adjusted so as to minimize the R factor. The fractional populations of the gt and tg forms were soon found to be negligible for the ribose ring in the C3'-endo form so that these population parameters were discarded in the subsequent adjustment. By the method of least squares, the most probable values (and standard deviations) of the ϕ' angles and fractional populations were obtained as shown in Figure 4. The standard deviations of variable parameters are all reasonably small. The calculated values of vicinal spin-coupling constants, shift ratios, and relaxation ratios (calculation III in Table I and II) agree satisfactorily with the observed values, and the R factor is now reduced as small as 0.10 so that solution III is an acceptable one. The internal rotation angles about the C3'-O3' bond were adjusted to $\phi' = 281 \pm 11^\circ$ for the G^+ form and $211 \pm 8^\circ$ for the G^- form. These values are appreciably different from the conventional values (300° and 180°) for staggered rotamers. Furthermore, the interrelations between the fractional populations of adjacent flexible sites are now quantitatively elucidated. The ribose ring in the C3'-endo form exclusively takes the G^- form about the C3'-O3' bond and the gg form about the C4'-C5' bond. On the other hand, the ribose ring in the C2'-endo form preferentially takes the G^+ form about the C3'-O3' bond and the tg and gg forms about the C4'-C5' bond.

Confidence Check. In the final stage of model refinements, the interrelations were taken into account among the population parameters of various flexible sites, including the interrelation between the internal rotation about the C3'-O3' bond and O3'-P bond. For any model systems thus tested, however, the R factor was not reduced by the direct search method, and the standard deviations of variable parameters were not improved. Accordingly, solution III (Figure 4) is the most reasonable one, and any further interrelation among flexible sites need not be incorporated as far as the observed data of the vicinal coupling constants, lanthanide-induced shift ratios, and relaxation ratios of Up are concerned.

Discussion

Advanced Features of Computer Program COFLEM. In the previous computer program PCS-II (Inagaki et al., 1978), the fractional populations of overall conformers were taken up as

variable population parameters together with appropriate structure parameters. However, for the molecule with many flexible sites (such as Up), the number of overall conformers possible is excessively large, and their fractional populations may not be uniquely determined from the observable data available. Accordingly, in the advanced lanthanide-probe analysis with the computer program COFLEM, the overall conformer equilibrium is expressed as combinations of local conformational equilibria for flexible sites. Thus, the interrelations among local conformational equilibria for adjacent flexible sites can be explicitly taken into account. On the other hand, the interrelation between two flexible sites, which are far from each other in the molecule, may be neglected in the conformation search, so that the number of adjustable parameters can be kept reasonably small. Thus, the most probable values of population and structure parameters are uniquely determined by the method of least squares, as demonstrated for the Up molecule under Conformation Search. Furthermore, in the program COFLEM, a highly improved algorithm is used so that the convergence of variable parameters is now quite fast in the minimization of R factor. This allows us to make a variety of trials for the refinement of models (conformation trees). The calculation of standard deviations of variable parameters was already incorporated in the previous program PCS-II. This calculation process is also incorporated in the program COFLEM and is really indispensable for refinements of models and for assessing the reliability of population and structure parameters finally obtained.

The advanced lanthanide-probe method with COFLEM may be extended to include the treatment of nuclear Overhauser effects, so that the syn-anti equilibria about glycosidic bonds may also be included in the analyses of conformation equilibria. The advanced lanthanide-probe analysis is also applicable to molecules with many flexible sites, such as dinucleoside monophosphates, as soon as an appropriate number of observable data are available.

Local Conformation about O3'-P Bond. In the lanthanide-probe analysis of adenosine 3'-monophosphate, Geraldès (1979) assumed free rotation about the O3'-P bond. However, the results of the X-ray studies (Sundaralingam, 1973) and conformation energy calculations (Saran et al., 1972) indicate the presence of three rotamers (g^+ , t , and g^- ; see Figure 2), the g^+ and g^- forms being more stable than the t form. In fact, in the lanthanide-probe analysis of the conformational equilibrium of pU, Inagaki et al. (1978) have found that the three-rotamer model (g^+ 45%, t 18%, g^- 37%) is preferable to the free-rotation model. Similarly, in the present study on Up, the three-rotamer model for the O3'-P bond is found to be reasonable; the fractional population of the trans form (t $12 \pm 4\%$) is lower than those of the gauche forms (g^+ $53 \pm 11\%$, g^- $35 \pm 9\%$) (Figure 4). It may be noted that the fractional populations of the three rotamers about the O3'-P bond of Up are nearly the same as those of the three rotamers about the P-O5' bond of pU.

Interrelation between Ribose-Ring Puckering and Internal Rotation about the C4'-C5' Bond. From the comparison of ^1H - ^1H vicinal coupling constants of a number of nucleosides and nucleotides, the interdependency has been found between the ribose-ring puckering and the internal rotation about the C4'-C5' bond (Hruska, 1973; Davies, 1978a,b). In fact, such an interrelation has been elucidated quantitatively by the lanthanide-probe analysis of the conformational equilibrium of pU (Inagaki et al., 1978). In the lanthanide-probe analysis of adenosine 3'-monophosphate (Geraldès, 1979), this interdependency has not been taken into account. However, in the

present lanthanide-probe analysis of the conformational equilibrium of Up with the computer program COFLEM, the interrelation between the ribose-ring puckering and internal rotation about the C4'-C5' bond is now quantitatively elucidated; the ribose ring in the C3'-endo form exclusively takes the gg form about the C4'-C5' bond while the ribose ring in the C2'-endo form preferentially takes the gg and gt forms (see Figure 4).

Local Conformations about the C3'-O3' Bond. The vicinal spin coupling constants $J(\text{C2}',\text{P})$ and $J(\text{C4}',\text{P})$ (Smith et al., 1973) and $J(\text{H3}',\text{P})$ (Davies, 1978a) may be used for studying the fractional populations of the three staggered rotamers with normal internal-rotation angles ($\phi' = 300^\circ$, 60° , and 180° for G^+ , T , and G^- , respectively). However, the fractional populations of these rotamers calculated from $J(\text{C2}',\text{P})$ and $J(\text{C4}',\text{P})$ entirely disagreed with those calculated from $J(\text{H3}',\text{P})$ (Davies, 1978a). Then an approximate method was proposed by Lee et al. (1976); only the G^+ and G^- forms were taken into account (the T form was neglected), and the dihedral angles ($\text{H3}'\text{-C3}'\text{-O3}'\text{-P}$) of the two forms were assumed to be equal in magnitude but opposite in sign. Thus, the magnitude of this dihedral angle was obtained as about 35° from the vicinal coupling constant $J(\text{H3}',\text{P})$. Later, the internal-rotation angles of the two gauche forms were set as 270° (G^+) and 210° (G^-), and, from the vicinal coupling constants $J(\text{C2}',\text{P})$ and $J(\text{C4}',\text{P})$, the fractional populations of the two forms were obtained as 48% (G^+) and 52% (G^-) (Alderfer & Ts'o, 1977). In our present lanthanide-probe (and spin coupling) analysis of the conformational equilibrium of Up, the fractional population of the T form about the C3'-O3' bond was in fact found to be negligible. The most probable values (and standard deviations) of the internal-rotation angles were obtained as $\phi' = 281 \pm 11^\circ$ for the G^+ form and $211 \pm 8^\circ$ for the G^- form. These ϕ' angles are consistent, within the standard deviations, with the dihedral angles ($\text{H3}'\text{-C3}'\text{-O3}'\text{-P}$) of about 35° as derived from $J(\text{H3}',\text{P})$ only. Thus, these ϕ' angles for the C3'-O3' bond (different from the conventional values of 300° and 180°) are now confirmed by the lanthanide-probe analysis (with the computer program COFLEM).

Interrelation between Ribose-Ring Puckering and Internal Rotation about the C3'-O3' Bond. From the steric considerations, the interdependency between the ribose-ring puckering and the internal rotation about the C3'-O3' bond was predicted (Lee et al., 1976; Davies, 1978a) and was qualitatively supported by the analyses of the long-range coupling constant $J(\text{H2}',\text{P})$ (Ezra et al., 1977). In fact, for the molecule of Up, such interrelation was quantitatively elucidated by the present lanthanide-probe (and spin coupling) analysis of the conformational equilibrium. As shown in Figure 4, the ribose ring in the C2'-endo form preferentially takes the G^+ form while the ribose ring in the C3'-endo form exclusively takes the G^- form. This interrelation is probably due to the steric interaction between the phosphate group and the ribose moiety. From the fractional population of the G^+ and G^- forms for the C2'-endo and C3'-endo forms and of the C2'-endo and C3'-endo forms themselves, the fractional populations of the four combinations (Figure 5) may be obtained as $38 \pm 12\%$ (C2'-endo- G^+), $16 \pm 12\%$ (C2'-endo- G^-), $1 \pm 2\%$ (C3'-endo- G^+), and $45 \pm 5\%$ (C3'-endo- G^-). For the C2'-endo- G^+ form, the steric repulsion between the phosphate group and the C4'-H4' bond appears to be as slight as the steric repulsion between the phosphate group and the C2'-H2' bond for the C3'-endo- G^- form (Figure 5). Accordingly, it is reasonable to find that these two forms are nearly equally predominant. On the other hand, for the C2'-endo- G^- form, some steric

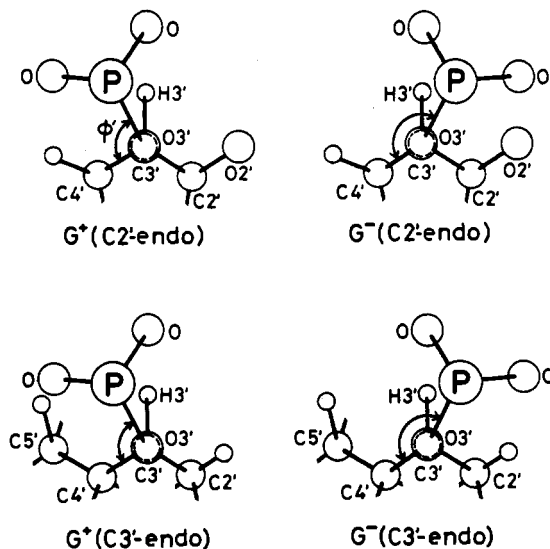


FIGURE 5: G^+ and G^- forms about the $C3'-O3'$ bond of ribose 3'-phosphate moiety in the $C2'$ -endo and $C3'$ -endo forms.

repulsion is expected between the phosphate group and the $C2'-O2'$ bond (Figure 5), and, in fact, the fractional population of this form is as low as $16 \pm 12\%$. Finally, the fractional population of the $C3'$ -endo- G^+ form (Figure 5) is negligible because of the strong steric repulsion between the phosphate group and the $C4'-C5'H_2$ group.

Short-Range Conformational Interrelations of Ribose 3'-Phosphate Moiety. Previously, Sarma & Danyluk (1977) proposed that in aqueous solution the conformational preferences about various bonds in nucleotides are interdependent and perturbation of the geometry along a key bond in the backbone causes a series of internal conformational adjustments similar to domino effects. Davies (1978b) also stated that analysis of the interrelationships between the conformational properties of adjacent bonds of nucleotidyl units provides insight into the cooperative nature of conformation of nucleic acids. However, for such investigations, analyses only of spin coupling constants are not conclusive. In the present study, however, the short-range conformational interrelations of the ribose 3'-phosphate moiety are now quantitatively elucidated by the lanthanide-probe analyses on monoanionic Up molecule, a good model for ribonucleotide units of RNA with un-ionized base and monoanionic phosphate group. The ribose ring in the $C3'$ -endo form exclusively takes the G^- form about the exocyclic $C3'-O3'$ bond and the gg form about the $C4'-C5'$ bond, thus inducing inflexibility of ribonucleotide units. This gg - $C3'$ -endo- G^- form of the ribose 3'-phosphate moiety is the basic structural unit in the base-stacked ordered conformation of the RNA A type helix. On the other hand, the ribose ring in the $C2'$ -endo form takes more than one local conformation about the $C3'-O3'$ bond ($G^+ > G^-$) and about the $C4'-C5'$ bond ($gg, gt > tg$), and thus the ribose 3'-phosphate moiety with the $C2'$ -endo ribose ring is more flexible than that with the $C3'$ -endo ribose ring. Such short-range conformational interrelations of the ribose 3'-phosphate moiety possibly affect the dynamic structural feature of ribonucleic acid in solution.

Ribose-Ring Puckering in Helical RNA Chains. Bolton & James (1979, 1980a,b) have found that the A-type helix of RNA has internal motions of the order of nanoseconds, somewhat slower than those of the B-type helix of DNA. Hogan & Jardetzky (1979) have proposed a model that the internal motion of DNA B type helix is caused by the transition between the $C2'$ -endo and $C3'$ -endo forms, concomitant with the resultant rearrangements in the backbone conformation.

In this connection, it is interesting to inquire whether such ribose-ring puckering is likely in RNA helix. In the present study of Up, the fractional population of the $C2'$ -endo- G^- form ($\sim 15\%$) is found to be much lower than that of the $C3'$ -endo- G^- form ($\sim 45\%$) but is not negligible (the G^- form is obviously more favorable to the base stacking with the 3' neighbor than is the G^+ form). Such accessibility to the $C2'$ -endo- G^- form will allow some motions of the ribose phosphate moiety while the base stacking in RNA chains is retained.

Reinforcement of RNA Structures through Stabilization of the $C3'$ -Endo Form. The RNA A type helical structure may be remarkably reinforced by stabilizing the $C3'$ -endo form of ribonucleotide units; the $C3'$ -endo ribose ring almost exclusively takes the gg form and G^- form about the exocyclic $C4'-C5'$ and $C3'-O3'$ bonds, respectively, because of the short-range conformational interrelations. For example, the gg - $C3'$ -endo form is highly stable in 2-thiopyrimidine nucleosides (and their 5'-mononucleotides), probably due to the steric interaction between the 2-thiocarbonyl group and the 2'-OH group (Yokoyama et al., 1979a). Because of such conformational stability, polynucleotide chains having 2-thiopyrimidine nucleosides are much more thermostable than those having pyrimidine or 4-thiouracil nucleosides (Yokoyama et al., 1979a, and references therein). Similarly, naturally occurring 2-thiouridine nucleotides located in specific sites of tRNAs probably contribute to the thermostability (Watanabe et al., 1979) and correct codon recognition (Yokoyama et al., 1979a), again because of the conformational stability of the ribose 3'-phosphate moiety with the $C3'$ -endo ribose ring.

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