^{31}P Magnetic Relaxation in Polynucleotides*

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Phosphorus-31 nuclear spin-lattice relaxation times and nuclear Overhauser enhancement upon nonselective proton saturation were measured on polyriboadenylic acid, polyribocytidylic acid, and polyribouridylic acid. A method is described to separate dipolar contribution from the contribution of chemical shift anisotropy for the phosphorus relaxation by the combined use of T_1 and NOE data. Chemical shift anisotropies ($\delta_{//}-\delta_{\perp}$) of phosphorus in these polynucleotides in solution are estimated to be in the range 120—165 ppm. The molecular motion of the phosphorus moiety becomes faster in the order, poly(A) < poly(C) < poly(U), the thermal activation processes being characterized by single activation energies, *i.e.*, 5.1, 5.3—6.0, and 7.2—8.1 kcal/mol for poly(U), poly(C), and poly(A), respectively.

Phosphorus nucleus plays a key role in the main chain structure of nucleic acids. Its relatively large nuclear moment with its 100% natural abundance makes the ^{31}P nucleus a good sample for nuclear magnetic resonance studies. Numerous reports are available on ^{31}P magnetic resonance of nucleic acid systems, but most of them are based on the analysis of chemical shift[1] and spin-spin coupling constant.[2] Studies of nucleic acids by ^{31}P relaxation measurements have only recently begun, and their relaxation mechanisms and use for the study of nucleic acid structures and dynamics have not been investigated in detail.

Akasaka studied dynamic structure of polyriboadenylic acid using ^{31}P relaxation assuming solely dipolar mechanism for relaxation.[3] Recently Hayashi *et al.* applied ^{31}P relaxation to studies of structures of yeast transfer RNA$^{\mathrm{phe}}$ based on more rigorous analysis of ^{31}P relaxation including the contribution from chemical shift anisotropy.[4] In the present paper, a detailed account is given on the method of analysis of ^{31}P relaxation as well as the results of its application to single-stranded polyribonucleotides.

Experimental

Materials. Homopolyribonucleotides (poly(A), poly(C), and poly(U)) (M_r > 100,000) from Sigma Chemical Company were used. They were dialyzed extensively in 0.1 M NaCl, mainly to remove paramagnetic metal ions. Samples for ^{31}P NMR measurements were prepared in concentrations of 60—90 mM (in monomer unit) in 99.75% D_2O containing a small amount of EDTA ([EDTA]/[P] = 1/20). All the samples were throughly degassed on a vacuum line before NMR measurements.

ApU (from Sigma) was treated with chelex and then prepared for NMR measurements as in the case of polynucleotides.

Methods. Measurements of ^{31}P spin-lattice relaxation times (T_1) were performed on a JEOL PFT-100 Fourier transform NMR spectrometer operating at 40.48 MHz under proton noise irradiation, by utilizing 180°-t-90° (inversion recovery) pulse sequences.

Nuclear Overhauser enhancement of ^{31}P resonance upon proton irradiation was obtained as relative integrated intensity of ^{31}P resonance between conditions with the proton noise-decoupler on and off by using sodium pyrophosphate as an internal standard. The proton decoupling power was kept less than 10 watts to avoid temperature rise in the samples.

Analysis of ^{31}P Relaxation

Three major mechanisms are considered to be important in determining ^{31}P longitudinal relaxation in nucleic acids:
(1) dipolar interaction with neighbouring protons, (2) chemical shift anisotropy, and (3) interaction with paramagnetic ions. These interactions are coupled with molecular motions to cause phosphorus relaxation in solution, and must be separated in order to utilize ^{31}P relaxation for a quantitative study of nucleic acid structures and dynamics.

First, the contribution from paramagnetic impurities and dissolved oxygen can be removed almost throughly (see Experimental). The absence of paramagnetic effects can be verified by observation of large nuclear Overhauser enhancements.

Separation of the dipolar contribution from the chemical shift anisotropy contribution is more intricate, requiring inspection into the nature of the relaxation mechanisms. One way frequently used on phospholipid systems[5] is to measure line widths at more than one observing frequency. This is based on the fact that, while the magnitude of the dipolar interaction is independent of the observing frequency ω, that of the chemical shift anisotropy depends on ω^2.[6] However, often in ^{31}P line width, contribution from the component that is linearly dependent ω can not be neglected, leading to errors in the evaluation of chemical shift contribution. Moreover, the availability of ^{31}P NMR spectrometers for a few to several different frequencies can not be usually expected.

In the present work, we have taken an alternative approach for separating two mechanisms, utilizing nuclear Overhauser enhancement of ^{31}P signals upon saturation of proton signals. The utility of the method lies in the fact that the nuclear Overhauser effect occurs only in the case of dipolar relaxation with protons, but not in the case of chemical shift anisotropy relaxation.

Following three basic assumptions are made. (1) The molecular motion of the phosphorus moiety can be approximated as isotropic and is therefore characterized by a single correlation time τ_c. (2) The same correlation time τ_c can be used in common for both the dipolar relaxation and the chemical shift anisotropy relaxation. (3) The geometrical as well as the electronic structure of the phosphorus moiety is invariant in the measured

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temperature range so that the distances between the phosphorus atom and the neighbouring protons (*e.g.*, $H_{3'}$, $H_{5'}$, and $H_{5''}$) as well as the anisotropy in the phosphorus chemical shift can be treated as temperature independent quantities.

Assumptions (1) and (2) are expected from simple approaches in NMR theory[6], and can be allowed as a first step. On the other hand, (3) might not be justified easily in a relatively flexible polymer such as a single-stranded polynucleotide. However, as an example, in the case of ApA and UpU phosphorus-proton coupling constants, $^3J_{PH_5'}$, $^4J_{PH_4'}$, and $^3J_{PH_3'}$, show only small variations (less than 10%) in going from 20 °C to 80 °C,[7] indicating relative invariance of rotamer populations about the $C_{3'}$—$O_{3'}$ and $C_{5'}$—$O_{5'}$ bonds with temperature. Unfortunately, no such data are available for polynucleotides, but the variations in rotamer populations might be expected to be even smaller in polynucleotides. In view of this, assumption (3) is considered to be reasonable.

Based on assumptions (1) and (2), the time dependence of the longitudinal magnetization $\langle I_Z\rangle_P$ of phosphorus, in the absence of relaxation contribution from paramagnetic impurities, should obey the equation[8]

$$\frac{\mathrm{d}\langle I_Z\rangle_P}{\mathrm{d}t} = (\sum_i \rho_{PH_i} + \rho_P)(I_{0P} - \langle I_Z\rangle_P) + \sum_i \sigma_{PH_i}(I_{0H_i} - \langle I_Z\rangle_{H_i}), \tag{1}$$

where I_{0P} and I_{0H_i} denote the magnetization of phosphorus and the i-th proton, respectively, at thermal equilibrium with the lattice, and $\langle I_Z\rangle_P$ and $\langle I_Z\rangle_{H_i}$ the magnetization of phosphorus and i-th proton, respectively, at time t after application of a 180° pulse.

ρ_{PH_i} and σ_{PH_i} are relaxation terms due to dipolar interaction between the phosphorus nucleus and the i-th proton, and expressed by

$$\rho_{PH_i} = \frac{\gamma_P^2\gamma_H^2\hbar^2}{r_i^6}\left\{\frac{1}{10}\frac{1}{1+(\omega_P-\omega_H)^2\tau_c^2} + \frac{6}{10}\frac{1}{1+(\omega_P+\omega_H)^2\tau_c^2} + \frac{3}{10}\frac{1}{1+\omega_P^2\tau_c^2}\right\}\tau_c, \tag{2}$$

$$\sigma_{PH_i} = \frac{\gamma_P^2\gamma_H^2\hbar^2}{r_i^6}\left\{-\frac{1}{10}\frac{1}{1+(\omega_P-\omega_H)^2\tau_c^2} + \frac{6}{10}\frac{1}{1+(\omega_P+\omega_H)^2\tau_c^2}\right\}\tau_c, \tag{3}$$

where r_i represents the distance between the phosphorus atom and the i-th proton, ω_P and ω_H are the Larmor angular frequencies for phosphorus and proton, respectively. ρ_P is the relaxation term due to chemical shift anisotropy and given by[6]

$$\rho_P = \frac{2}{15}(\delta_{//}-\delta_\perp)^2\omega_P^2\frac{\tau_c}{1+\omega_P^2\tau_c^2}, \tag{4}$$

where axial symmetry is assumed for the chemical shift tensor.

Under saturation of all the proton resonances due to proton-noise irradiation, $\langle I_Z\rangle_{H_i}=0$ for all i and Eq. 1 can be solved, giving

$$\langle I_z\rangle_P = I_{0P}\left(1+\frac{\sum_i \sigma_{PH_i}}{\sum_i \rho_{PH_i}+\rho_P}\cdot\frac{\gamma_H}{\gamma_P}\right)(1-2e^{-t/T_1}), \tag{5}$$

with

$$\frac{1}{T_1} = \left(\frac{1}{T_1}\right)_{\text{dipole}} + \left(\frac{1}{T_1}\right)_{\text{c.s.a.}} = \sum_i \rho_{PH_i} + \rho_P$$
$$= A\left\{\frac{1}{10}\frac{1}{1+(\omega_P-\omega_H)^2\tau_c^2} + \frac{6}{10}\frac{1}{1+(\omega_P+\omega_H)^2\tau_c^2} + \frac{3}{10}\frac{1}{1+\omega_P^2\tau_c^2}\right\}\tau_c + B\cdot\frac{\tau_c}{1+\omega_P^2\tau_c^2}, \tag{6}$$

where

$$A = \sum_i \frac{\gamma_P^2\gamma_H^2\hbar^2}{r_i^6}, \tag{7}$$

$$B = \frac{2}{15}\omega_P^2(\delta_{//}-\delta_\perp)^2. \tag{8}$$

The fractional increase (η) of phosphorus magnetization upon saturation of proton signals, *i.e.*, the nuclear Overhauser enhancement, is given by

$$\eta = \frac{\sum_i \sigma_{PH_i}}{\sum_i \rho_{PH_i}+\rho_P}\cdot\frac{\gamma_H}{\gamma_P} = \frac{2.47\sum_i \sigma_{PH_i}}{\sum_i \rho_{PH_i}+\rho_P} \tag{9}$$

Under conditions of extreme narrowing $((\omega_P+\omega_H)^2\tau_c^2 \ll 1)$, Eqs. 6 and 9 become

$$\frac{1}{T_1} = (A+B)\tau_c, \tag{10}$$

$$\eta = 1.235\left(\frac{A}{A+B}\right). \tag{11}$$

If we may assume that the average molecular conformation of the ribose-phosphate moiety does not change significantly with temperature (assumption (3)), we may regard A and B as parameters independent of temperature.

$1/T_1$ expressed by Eq. 6 goes through a maximum (or T_1 goes through a minimum) at a certain value of τ_c ($\tau_c \simeq \omega_P^{-1}$), the particular value of which depending on the observing frequency ω_P and the relative values of A and B. On the other hand, the nuclear Overhauser enhancement η, which is nearly zero or very small for $\tau_c > \omega_P^{-1}$ (see Eq. 9), increases monotonically with the decrease of τ_c to a final value given by Eq. 11. Such behavior of T_1 and η is illustrated in Fig. 1, as a function of τ_c for three representative cases, $B=0$, $A/B=1$, and $A=0$.

Information Obtainable from ^{31}P Relaxation and Nuclear Overhauser Enhancement

In practice, if we know the relative values of A and B from measurements obtained under extreme narrowing conditions (Eq. 11), we can determine the τ_c value at the minimum point of T_1 by using Eq. 6. Knowing both the τ_c value and the T_1 value at the minimum point of T_1, we can also determine from Eq. 6 the absolute values of A and B, which in turn, give information as to the effective phosphorus-proton distance, defined by

$$\bar{r}_{P-H} = (\sum_i 1/r_i^6)^{-1/6}, \tag{12}$$

and the magnitude of the effective ^{31}P chemical shift anisotropy $(\delta_{//}-\delta_\perp)$ in solution.

Since A and B values are fixed as temperature invariant quantities, the only unknown variable in the right-hand side of Eq. 6 is τ_c. From a measured

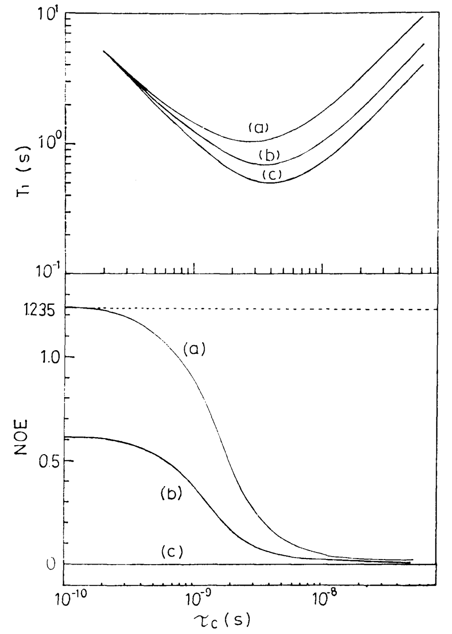

Fig. 1. Theoretical dependence of ^{31}P spin-lattice relaxation time (T_1) and the nuclear Overhauser enhancement (η) upon nonselective proton saturation on the rotational correlation time τ_c for three representative cases of relaxation mechanism:
(a) dipolar 100% ($B=0$ in Eq. 6), (b) dipolar 50%, chemical shift anisotropy 50% ($A/B=1$), (c) chemical shift anisotropy 100% ($A=0$). Isotropic molecular rotation is assumed and the resonance frequency of ^{31}P nucleus at 40.5 MHz is presumed.

value of T_1 at any desired temperature, we can determine τ_c, a quantity that characterizes the molecular motion around the phosphorus moiety.

Application to Polynucleotides

Figure 2 shows the temperature dependence of spin-lattice relaxation time (T_1) for phosphorus in three polyribonucleotides, poly(U), poly(C), and poly(A), in single-stranded forms in neutral D_2O solutions. Data for poly(A), taken from a previous work[3], are subjected to a more rigorous treatment here. Poly(G) behaves in a distinctly different manner from other polynucleotides, showing neither T_1 minimum nor nuclear Overhauser enhancement ($\omega_p^2\tau_c^2 \gg 1$) because of its unique multi-stranded structure. We notice the following from Fig. 2,
(1) Each polynucleotide shows a minimum value of T_1 in the temperature range of measurement so that the method of analysis described above can be applied.
(2) The temperature at which $T_{1\,\mathrm{min}}$ occurs increases in the order, poly(U)<poly(C)<poly(A), showing that the rate of molecular rotation around the phosphorus atom decreases in this order.

The lower part of Fig. 2 shows the corresponding nuclear Overhauser enhancement (η) for these polynucleotides. We see that:
(3) Nuclear Overhauser enhancement at the highest observed temperature ($\eta=0.6$—1.0) is greater than 50% of that ($\eta=1.235$) expected for the case of 100% dipolar relaxation under extreme narrowing conditions ($B=0$ in Eq. 11), although except for poly(U), the systems may not be under extreme narrowing conditions even at the highest observed temperatures.

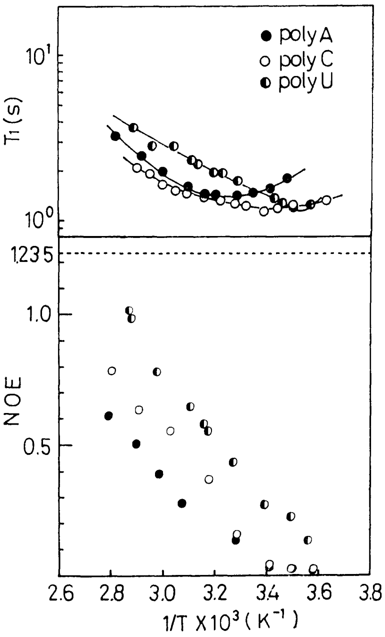

Fig. 2. Temperature dependence of ^{31}P spin-lattice relaxation times and ^{31}P nuclear Overhauser enhancement (η) upon nonselective proton saturation of poly U, poly C, and poly A in neutral D_2O solutions (pH$=6.0+0.1$, [Na^+]$=0.1$ M). Concentration of polynucleotides are 92 mM for poly U, 73 mM for poly C, and 60 mM for poly A (monomer unit).

(4) At any temperature, the nuclear Overhauser enhancement decreases in the order, poly(U)>poly(C)>poly(A), in line with the rate of molecular motion estimated above from the T_1 data.

Observation (3) shows that $A/(A+B)>0.5$ holds for the three polynucleotides, and the dipolar mechanism is a dominant source of ^{31}P longitudinal relaxation at 40.48 MHz. To ensure measurement of nuclear Overhauser effect under extreme narrowing conditions, a model compound of lower molecular weight ApU was investigated, which indicated $\eta=1.0$.

By using the maximum values of nuclear Overhauser enhancement in the measured temperature range, and the T_1 and τ_c values at $T_{1\,\mathrm{min}}$, the values or the ranges for A and B are determined in each polynucleotide, and the corresponding values or the ranges for $\bar{r}_{\mathrm{P-H}}$ and $(\delta_{//}-\delta_{\perp})$ are estimated, according to the method of analysis described. The values are summarized in Table 1. The corresponding quantities for yeast transfer RNA$^{\mathrm{Phe}}$, previously obtained from a similar analysis[4] are given for comparison.

The relatively small variation of $\bar{r}_{\mathrm{P-H}}$ values among different polynucleotides seems to indicate that conformational variation is small among the backbone structures of these polynucleotides. The values of chemical shift anisotropy $(\delta_{//}-\delta_{\perp})$ were obtained in the range 120—160 ppm. Differences in $(\delta_{//}-\delta_{\perp})$ values between different polynucleotides can not be discussed in view of the limited accuracy of the present method

TABLE 1. SUMMARY OF ^{31}P RELAXATION DATA AND MOLECULAR PARAMETERS DEDUCED THEREFROM IN HOMOPOLYRIBONUCLEOTIDES AND RELATED SUBSTANCES

Nucleotide	$(T_1)_{\min}$ (s)	NOE	Dipolar contribution (%)	$\bar{r}_{\text{P-H}}^{\text{a)}}$ (Å)	$\delta_{//}-\delta_{\perp}$ (ppm)	E_a (kcal/mol)
poly(U)	1.18	2.0 (72 °C)	$\simeq$ 80	2.33	128	5.1
poly(C)	1.15	1.8 (83 °C)	$>$ 65	2.1—2.4	$<$165	5.3—6.0
poly(A)	1.41	1.6 (83 °C)	$>$ 50	2.2—2.6	$<$158	7.2—8.1
(helix)	1.55			2.49	130	
$\text{tRNA}^{\text{phe b)}}_{\text{yeast}}$		1.9 (80 °C)	$\simeq$ 73			
(random)	1.80			2.25	120	6.0 (45—80 °C)
ApU$^{\text{c)}}$		2.0 (79 °C)	80			

a) Effective P-H distance; $1/\bar{r}_{\text{P-H}}^6=\sum_i(1/r_i^6)$. b) Data taken from Ref. 4. c) Measured at 20 mM.

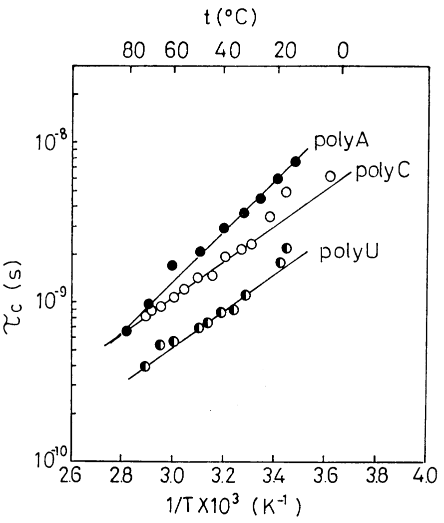

Fig. 3. Temperature dependence of rotational correlation times (τ_c) of poly U, poly C, and poly A as deduced from T_1 and NOE data of Fig. 2.

although the isotropic value of the chemical shift definitely varies.$^{9)}$ Extreme narrowing conditions are insufficiently fulfilled for poly(A) and poly(C). On the other hand, ApU which is certainly under extreme narrowing conditions in all the temperature ranges measured shows $\eta \simeq 1.0$ or 80% dipolar contribution. By taking account of these facts, we expect that if measurements were done under extreme narrowing conditions for poly(A) and poly(C), the value of η would become as large as that of ApU. Then a probable value of $(\delta_{//}-\delta_{\perp})$ in single stranded polynucleotides in an aqueous solution can be placed in the lower side of the range, *i.e.*, 120—130 ppm.

Furthermore, the τ_c value is obtained as a function of temperature for each polynucleotides (Fig. 3).

It is to be noted that τ_c follows approximately the following equation:

$$\tau_c^{-1} = \tau_0^{-1}\exp(-E_a/RT) \qquad (13)$$

where E_a is the activation energy. The fact that these single-stranded polynucleotides approximately follow a single activation process indicates that their thermal melting is non-cooperative, *i.e.*, the motional activation occurs in a non-cooperative fashion. This makes a sharp contrast with the result for tRNA, which shows a few distinctive steps of activation process.$^{4)}$

The τ_c values observed are in the order of 10^{-9} s and can be considered to represent not the whole motion of the molecule ($M_r>100{,}000$) but the local torsional motions around the sugar phosphate backbone. Consequently, the E_a values are considered to represent primarily the torsional barriers around the sugar-phosphate back bone. The observation that the E_a value increases in the order poly(U)<poly(C)<poly(A) seems to reflect the fact that the incressing tendency of base-stacking gives additional barriers for the molecular motion of the sugar-phosphate moiety.

Conclusion

Important conclusions obtained by inspection of Table 1 and Figs. 2 and 3 may be summarized as follows.

1) The relaxation mechanisms are dipolar dominant (more than 50% in the extreme narrowing region) at this observing frequency (40.48 MHz).

2) Conformations involving the phosphorus atom and the neighbouring protons do not seem to vary significantly between different polynucleotides, as judged from $\bar{r}_{\text{P-H}}$ values.

3) Phosphorus chemical shift anisotropy, $(\delta_{//}-\delta_{\perp})$, of single-stranded polynucleotides in solution is in the range 120—165 ppm. It would probably range around 120—130 ppm if measurements could all be made under extreme narrowing conditions.

4) Molecular motions around the phosphate group of single-stranded homopolyribonucleotides become faster in the order, poly(A)<poly(C)<poly(U). The thermal activation is characterized by single activation energies, 5.1, 5.3—6.0, and 7.2—8.1 kcal/mol for poly(U), poly(C), and poly(A), respectively. These results seem to imply that the base-stacking plays an important role in restricting flexibility of the main chain structure of single-stranded polynucleotides.

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