Determination of Structures of Molecular Complex in Solution Using Unpaired Electron-Induced Nuclear Magnetic Relaxation. Application to Adenosine 5′-Monophosphate and *N*-Methylphenazinium Cation Radical

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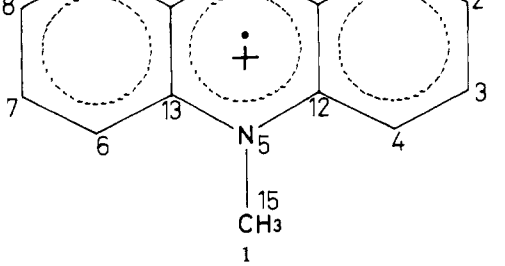
Abstract: A procedure is described by which a structure of molecular complex in solution is found when one of the interacting molecules is a free radical. The method is based on measurements of unpaired-electron induced nuclear magnetic relaxation, and can apply to a system involving a free radical whose unpaired electron is delocalized over the molecular framework. The method was first applied to a system of adenosine 5′-monophosphate (5′-AMP) and *N*-methylphenazinium cation radical ($MPH^+\cdot$) in a neutral D_2O solution. The experimental procedures are described to obtain the association constant, the rotational correlation time, and the averaged electron-nuclear distances in the complex. A process of computer-assisted search for probable structures of the complex is described, which is based on the comparison of the experimental electron-nuclear distances with those calculated for essentially all possible mutual orientations of the two molecules and the internal rotations by taking the distribution of the unpaired electron in the free radical explicitly into account. It is shown that all allowed structures of the complex between 5′-AMP and $MPH^+\cdot$ are such that two π planes are nearly parallel with a plane-to-plane distance of 3.0–3.5 Å. The best agreement between the experimental and the calculated distances is obtained when 5′-AMP takes an anti conformation and the long axes of the two π planes of 5′-AMP and $MPH^+\cdot$ orient nearly perpendicular to each other. This orientation of the two rings was supported by the simple consideration of intermolecular forces.

Nuclear magnetic resonance (NMR) has been used extensively to study *intra*molecular structure of a molecule. In some cases, it can also be applied to probe structures of molecular complexes in solution through the chemical shift analysis.[1] Direct estimate of internuclear distances through the nuclear-nuclear Overhauser effect[2] or the method of DESERT (deuterium substitution effect on relaxation times)[3] often fails when applied to nuclei belonging to two different molecules forming an intermolecular complex, because the magnetic dipolar field of a nucleus is usually too small for this purpose. The use of an electron magnetic moment, instead of a nuclear magnetic moment, is therefore necessary in order to obtain sufficient intermolecular effects on nuclear relaxation. So far, however, quantitative treatment of unpaired electron-induced relaxation for structural elucidation has been limited to systems where the unpaired electron distribution can reasonably be approximated as a point in space, such as those containing paramagnetic metal ions.[4] Extension of such treatment to systems where one of the interacting molecules is a free radical whose unpaired electron is delocalized over the molecular framework appears important, in view of the fact that free-radical intermediates from various drugs, carcinogens,[5] and coenzymes[6] frequently appear in metabolic pathways.

In treating such systems, however, one has to take two new factors into consideration. First, we must employ three more parameters to define the mutual orientation of two rigid molecules in addition to the three spatial coordinates that define the relative positions of the representative points of the two molecules (e.g., the centers of the two molecules). In addition, one needs additional parameters to define intramolecular conformations of the interacting molecules when they have internal freedom of rotation. Secondly, we need an explicit knowledge of the distribution of unpaired electron in a free radical. In the present work, computer programs are developed which serve to find probable structures of a complex, taking the above factors explicitly into consideration.

There are some additional factors that must be considered carefully in a quantitative analysis of relaxation data of a system containing a free radical: (1) the limited stability of a free radical and the accurate determination of its concentration; (2) an accurate determination of the association constant in each individual case, because the association constant is usually small in these systems and all the radicals present are not bound with the diamagnetic ligand; (3) the determination of the rotational correlation time of the molecular complex.

In the present paper, we will show how we have overcome these difficulties and applied our computer programs to elucidate probable structures of a complex between a free radical and a diamagnetic ligand molecule, finding an example in a system of *N*-methylphenazinium cation radical ($MPH^+\cdot$, **1**) and adenosine 5′-monophosphate (5′-AMP).



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This free radical, $MPH^+\cdot$, appears as an intermediate product from phenazinium methosulfate (PMS) in living tissues.[7] PMS shows some mutagenic activity when applied to bacteriophage T4,[8] and its interaction with nucleic acids poses an interesting question with respect to a model of mutagenesis and carcinogenesis. An intercalative mode of binding has been suggested to occur between $MPH^+\cdot$, MP^+ (a diamagnetic cation of *N*-methylphenazinium), or the related free-radical derivative and DNA from equilibrium binding studies based on ESR and optical absorptions.[8-11] Some direct evidence for a similar mode of binding between $MPH^+\cdot$ and single-stranded polyadenylic acid[12] was previously presented

0002-7863/78/1500-0706$01.00/0

in a line of study similar to the present one that utilizes unpaired electron-induced nuclear magnetic relaxation as a structural probe. The present study aims at elucidation of some detailed structure of an intermolecular complex of $MPH^{+}\cdot$ with nucleic acid at a simpler nucleotide level where measurements of nuclear relaxation can be performed on all the nonexchangeable individual protons of the diamagnetic molecule.

Experimental Section

$MPH^{+}\cdot$ radicals are synthesized as perchlorate or oxalate crystals from phenazine methosulfate (obtained from Sigma) according to the method reported earlier.[13] Disodium salts of 5′-AMP (obtained from Kohjin) were used without further purification. For NMR measurements, AMP solutions (from 5 to 50 mM) containing ethylenediaminetetraacetic acid (from 1 to 2 mM) were lyophilized and then dissolved in D_2O. The lyophilization and the addition of D_2O were repeated twice to reduce the background HDO signal. The pH had been adjusted to 6.0 (as pH meter reading) before lyophilization. Crystalline salts of $MPH^{+}\cdot$ were added under vacuum to finally degassed AMP solutions and then sealed, because the $MPH^{+}\cdot$ radicals are easily oxidized to diamagnetic forms in the presence of oxygen.

Optical densities of $MPH^{+}\cdot$ at 388 and 446 nm were measured directly in an NMR sample tube of 5-mm diameter immersed in a water-filled optical cell (1 cm in length) within the error range of about 2%. Nuclear magnetic relaxation times of proton and phosphorus of 5′-AMP were measured at 26.0 ± 0.5 °C with a JEOL PS-100 NMR spectrometer equipped with a pulse Fourier transform unit, operating at 100 MHz for proton and 40.5 MHz for phosphorus. The longitudinal relaxation times (T_1) were measured by means of 180°-t-90° pulse sequences. ESR spectra were recorded on a JEOL PE-3X ESR spectrometer at X band.

Simulation of the structure of the molecular complex was carried out with a FACOM 230-75 computer and with a FACOM 230-35 system connected to a graphic display F6233A, in Kyoto University Data Processing Center.

Methods

A. Analysis of Relaxation Data in a System under Chemical Equilibrium. We assume a simple bimolecular association between 5′-AMP and $MPH^{+}\cdot$, i.e.,

$$\mathrm{AMP} + \mathrm{MPH}^{+}\cdot \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\mathrm{AMP\text{-}MPH}^{+}\cdot)_{\mathrm{complex}} \qquad (1)$$

The complexation of $MPH^{+}\cdot$ with dimeric (or higher aggregate) forms of 5′-AMP may be neglected in view of the low concentration (≤50 mM) of AMP used and the small value of the association constant of 5′-AMP ($K_{\mathrm{dimer}} = 0.9\ \mathrm{M}^{-1}$ at pH 6.0) which was determined from a separate measurement of chemical shifts. The dimerization of $MPH^{+}\cdot$ would also be neglected at the low concentration (from 0.02 to 0.1 mM) of $MPH^{+}\cdot$ used.[8]

In this situation, the longitudinal relaxation rate observed for a nucleus of AMP is given by[14]

$$\frac{1}{T_1} - \frac{1}{T_{10}} = \frac{1}{T_{1\mathrm{p}}} \qquad (2)$$

$$\frac{1}{T_{1\mathrm{p}}} = \frac{b}{T_{1\mathrm{b}} + \tau_{\mathrm{b}}} \qquad (3)$$

where T_1 and T_{10} are the longitudinal relaxation times in the presence and the absence of $MPH^{+}\cdot$, respectively, $1/T_{1\mathrm{p}}$ stands for the enhancement of the longitudinal relaxation rate by the presence of the small amount of $MPH^{+}\cdot$ radical, b is the fraction of AMP molecules bound with the radical in the total amount of AMP present, τ_{b} is the lifetime of AMP in the bound state ($\tau_{\mathrm{b}} = 1/k_{-1}$), and $T_{1\mathrm{b}}$ is the longitudinal relaxation time of a nucleus of AMP in the bound state. $1/T_{1\mathrm{b}}$ is related to the electron-nuclear distance ($\bar{r}$) by the relation[15]

$$\frac{1}{T_{1\mathrm{b}}} = \frac{3}{10}\gamma_{\mathrm{I}}^2 g^2\beta^2(\bar{r})^{-6}\frac{\tau_{\mathrm{c}}}{1+\omega_{\mathrm{I}}^2\tau_{\mathrm{c}}^2} + 2\pi^2A^2\frac{\tau_{\mathrm{e}}}{1+\omega_{\mathrm{s}}^2\tau_{\mathrm{e}}^2} \qquad (4)$$

where γ_{I} is the gyromagnetic ratio of the nucleus, $g\beta$ is the spin angular momentum of the electron, ω_{I} and ω_{s} are the Larmor angular frequencies of the nuclear and the electron spins, respectively, $\bar{r}$ is the reciprocal sixth-power-averaged distance between the unpaired electron and the nucleus, A is the isotropic hyperfine coupling constant in hertz, τ_{c} is the correlation time for the electron-nuclear magnetic dipolar interaction, and τ_{e} is the correlation time for the isotropic hyperfine interaction.

Since $\tau_{\mathrm{e}} \gg \omega_{\mathrm{s}}^{-1}$ holds in the present system as will be mentioned later, $T_{1\mathrm{b}}$ is dominated by the first term of eq 4. In addition, measurement of T_1 was made at various temperatures, which indicated that $T_{1\mathrm{p}}$ of all the protons increases with increasing temperature. This strongly suggests that $T_{1\mathrm{b}}$ is the dominant term in eq 3. Thus if we know the binding fraction b, we may obtain $T_{1\mathrm{b}}$ from eq 3 and therefore electron-nuclear distances ($\bar{r}$) in the AMP-$MPH^{+}\cdot$ complex from eq 4.

In order to estimate the binding fraction, the association constant K ($= k_1/k_{-1}$) must be known. Often, the association constant is obtained from the measurement of chemical shift as a function of concentration. In the present system in which a free radical with a relatively long electron spin relaxation time is involved and the lifetime of the complex is long, both the ring current shift and the paramagnetic shift are completely obscured by the broadening of the resonance line, due to the pronounced effect of the electron paramagnetism on the nuclear relaxation. In such a case, however, the electron-induced relaxation enhancement itself can be used as a means to elucidate the association constant, as shown below.

Under the condition that $[\mathrm{AMP}]_0 \gg [\mathrm{AMP\text{-}MPH}^{+}\cdot]$ and $T_{1\mathrm{b}} \gg \tau_{\mathrm{b}}$, eq 3 can be rewritten in the form

$$\frac{1}{T_{1\mathrm{p}}} = \frac{K[\mathrm{MPH}^{+}\cdot]_0}{T_{1\mathrm{b}}(1 + K[\mathrm{AMP}]_0)} \qquad (5)$$

or

$$T_{1\mathrm{p}}[\mathrm{MPH}^{+}\cdot]_0 = T_{1\mathrm{b}}[\mathrm{AMP}]_0 + T_{1\mathrm{b}}/K \qquad (6)$$

where $[\mathrm{AMP}]_0$ and $[\mathrm{MPH}^{+}\cdot]_0$ represent the total concentration of AMP and $MPH^{+}\cdot$ radical present. By measuring $T_{1\mathrm{p}}$ as a function of $[\mathrm{AMP}]_0$ and simultaneously measuring $[\mathrm{MPH}^{+}\cdot]_0$, one would be able to plot $T_{1\mathrm{p}}[\mathrm{MPH}^{+}\cdot]_0$ against $[\mathrm{AMP}]_0$, from which $T_{1\mathrm{b}}$ and K would be obtained simultaneously.

One important point not to be overlooked here is the fact that τ_{c} of 5′-AMP depends on the concentration of 5′-AMP itself[16] and therefore that $T_{1\mathrm{b}}$ is not a constant over the AMP concentration. Since 10 mM AMP is most frequently employed, we take 10 mM as a standard concentration. Because the system under consideration is in the extreme narrowing condition, $T_{1\mathrm{b}}$ at x mM of AMP can be estimated easily from that at 10 mM (designated by $T_{1\mathrm{b}}{}^{10}$) by factoring $\tau_{\mathrm{c}}{}^{10}/\tau_{\mathrm{c}}{}^{x}$, i.e., $T_{1\mathrm{b}}{}^{x} = (\tau_{\mathrm{c}}{}^{10}/\tau_{\mathrm{c}}{}^{x})T_{1\mathrm{b}}{}^{10}$. Hence eq 6 can be replaced by a more practical one,

$$T_{1\mathrm{p}}{}^{x}\left(\frac{\tau_{\mathrm{c}}{}^{x}}{\tau_{\mathrm{c}}{}^{10}}\right)[\mathrm{MPH}^{+}\cdot]_0 = T_{1\mathrm{b}}{}^{10}[\mathrm{AMP}]_0 + \frac{T_{1\mathrm{b}}{}^{10}}{K} \qquad (7)$$

By plotting the left-hand side of this equation with observed values of $T_{1\mathrm{p}}{}^{x}$, $\tau_{\mathrm{c}}{}^{x}/\tau_{\mathrm{c}}{}^{10}$, and $[\mathrm{MPH}^{+}\cdot]_0$, against the concentration of $[\mathrm{AMP}]_0$, constant parameters, $T_{1\mathrm{b}}{}^{10}$ and K, can be obtained simultaneously from the slope and the intercept.

Once knowing the association constant K, $T_{1\mathrm{b}}$ can alternatively be obtained from the slope of the plot of $1/T_{1\mathrm{b}}$ against the bound fraction b as indicated in eq 3. With a reasonably estimated value of τ_{c}, the elucidation of distance $\bar{r}_i{}^{\mathrm{exp}}$ between the various nuclei of 5′-AMP and the unpaired electron of $MPH^{+}\cdot$ is straightforward from eq 4.

B. Computer-Assisted Search for Probable Structures of the Complex. To search for probable structures of the complex

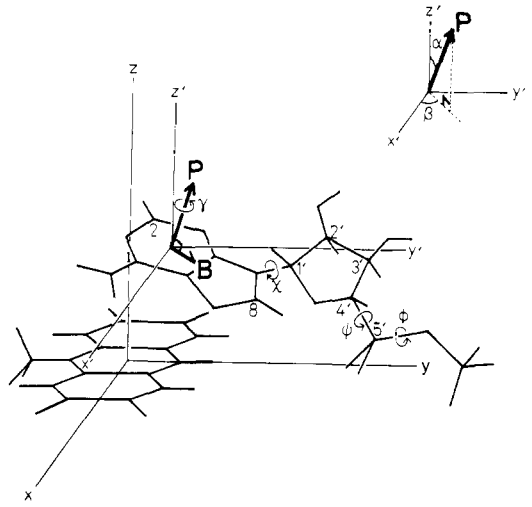

Figure 1. Coordinate systems used to represent the mutual orientation of 5′-AMP and $MPH^+\cdot$ and the intramolecular conformation of 5′-AMP. Arrow B with the length of 1.0 Å lies in the plane of the adenine ring.

based on the $\bar{r}_i^{exp}$ values, we generate all possible mutual orientations of the radical ($MPH^+\cdot$) and the diamagnetic ligand (5′-AMP) and calculate distances ($\bar{r}_i^{calcd}$) between the unpaired electron of $MPH^+\cdot$ and nucleus i of AMP. Mutual orientations which give $\bar{r}_i^{calcd}$ values in reasonable agreement with $\bar{r}_i^{exp}$ values are selected to be probable ones. In the actual calculation, values of $(\bar{r}_i^{calcd})^{-6}$ with $(\bar{r}_i^{exp})^{-6}$ are compared by the aid of computer programs, since these values have a linear relationship to the experimental error in T_1.

To represent the mutual geometrical relationship between any two rigid molecules, six parameters must be defined. As shown in Figure 1, the relative location of the center of the six-membered ring of 5′-AMP to the center of $MPH^+\cdot$ fixed at the origin are represented by Cartesian coordinates, x, y, and z. In addition, we need three additional parameters, α, β, and γ, to define the mutual orientation of the two molecules. The inclination of the adenine plane relative to the $MPH^+\cdot$ plane is represented by vector **P** which is taken perpendicular to the plane of the adenine ring. The direction of **P** is expressed by two angles, α and β, as defined also in Figure 1. The sixth parameter, γ, is a rotational angle about the vector **P**. These angles are equivalent to the usual Eulerian angles (β', α', and γ') with relations $\alpha = \alpha'$, $\beta = \beta' - 90°$, and $\gamma = \gamma' + \beta'$. Atomic coordinates of 5′-AMP[17] are available from x-ray diffraction data, and those of $MPH^+\cdot$ are the same as those of MP^+, also available from x-ray diffraction study[18] (see Table I).

To calculate averaged distances between protons of 5′-AMP and the unpaired electron, it must be considered that the unpaired electron is delocalized over the π-electron system of the $MPH^+\cdot$ molecule. The reciprocal sixth-power-averaged distance between nucleus i and the unpaired electron in eq 4 may be approximated by[19]

$$(\bar{r}_i^{calcd})^{-6} = \sum_j \frac{\rho_j}{2}\{(r_{ij})^{-6} + (r_{ij}')^{-6}\} \qquad (8)$$

where ρ_j is the spin density in the nucleus j of $MPH^+\cdot$, and r_{ij} and r_{ij}' are the distances between the observed nucleus i and two hypothetical points which are expected to represent the total distribution of the odd electron in the p_π orbital on nucleus j. These points are taken to be located at 0.7 Å above and below the plane of the $MPH^+\cdot$ molecule. This distance is given by adopting the p orbital of Slater type for a carbon atom. The spin density distribution of the $MPH^+\cdot$ radical is calculated by using the INDO computer program,[20] and is shown in Table I.

Table I. Atomic Coordinates[a] and the Spin Density[b] of $MPH^+\cdot$

Nucleus	x, Å	y, Å	ρ
C1	2.400	1.377	0.017
C2	3.590	0.705	0.049
C3	3.585	−0.710	0.019
C4	2.410	−1.320	0.010
N5	0.0	−1.392	0.399
N10	0.0	1.397	0.312
C11	1.180	0.714	0.059
C12	1.170	−0.714	0.024
C15	0.0	−2.930	0.000

[a] After C. J. Fritchie, Jr.[18] [b] From INDO calculation.

One must also consider that the diamagnetic ligand molecule such as 5′-AMP is not in general rigid, but that it has internal freedom of rotation. In the present case of 5′-AMP, we need an additional five parameters to calculate $(\bar{r})^{-6}$ for all the protons of 5′-AMP. It would take too much time, and in fact is impossible, to compare $(\bar{r}_i^{calcd})^{-6}$ with $(\bar{r}_i^{exp})^{-6}$ by independently changing all the 11 (6 + 5) variable parameters. Instead of doing this, probable intermolecular structures can be searched for in two steps by using two programs in succession.

The first program (program I) seeks for a mutual orientation of the two molecules for certain *fixed* internal rotations of 5′-AMP by iteration procedures. It is essentially composed of the linear minimization of the agreement function expressed by the combination of two functions. One of them is the normalized standard deviations[21] (NSD, weighted by experimental errors) of the expected values of $(\bar{r}_i)^{-6}$ from the corresponding experimental ones, which has the form

$$\text{NSD (\%)} = \sqrt{\frac{\sum_i (F_i^{calcd} - F_i^{exp})^2/{\sigma_i}^2}{\sum_i (F_i^{exp})^2/{\sigma_i}^2}} \times 100 \qquad (9)$$

where F_i represents $(\bar{r}_{H2})^{-6}$ or $(\bar{r}_i)^{-6}/(\bar{r}_{H2})^{-6}$ and σ_i stands for the experimental distribution of corresponding F_i for each observed nucleus i. H2 was taken as standard, because its relaxation rate was most enhanced by $MPH^+\cdot$. To reduce the influence of experimental errors, the value of $(1/{T_{1p}}^{10})_i/(1/{T_{1p}}^{10})_{H2}$ ($i \neq$ H2) was actually taken for F_i^{exp}. NSD of less than ~5% would be a good measure for a probable conformer, in view of the fact that average errors in T_1 measurements did not exceed 5% in the present experiment. The other is the summation of the proper switching functions, which takes a large value when the distance between two atoms is less than their van der Waals exclusive distances. The exclusive atomic radii for H, C, N, O, and the methyl group are taken to be 0.95, 1.4, 1.3, 1.2, and 1.3 Å, respectively. These values are obtained by multiplying 0.8 by the van der Waals radii and are expected to show repulsive energy greater than 0.5 kcal/mol for each atomic pair.[22]

Program I is written by FORTRAN, and the process of the computation is as follows. Two parameters out of x, y, and z, for example, x and y, are first fixed and all other four parameters, z, α, β, and γ, are varied for minimizing the agreement function from various initial values (z = 9.0 Å, α = 0 and 180°, β = arctan (y/x), γ = 0, 90, 180, 270°). In the present study, computations by this program I were carried out for six protons of 5′-AMP, i.e., H2, H8, H1′, H2′, H3′, and H4′, with the glycosidic torsional angle χ and the ribose puckering as adjustable parameters. The flow chart of the program I is shown in Figure 2.

The second program (program II) is interactive with a graphic display in which the complex structure is displayed on the scope with its numerical parameters and the values of the agreement function. Program II is also written by FORTRAN combined with basic graphic subroutines, and is arranged so

that any conformational parameter can be changed at our will. In the present study, program II was used to find ψ and ϕ angles (defined in Figure 1) that give reasonably small NSD values, based on the results obtained by program I.

Results and Discussion

In order to determine T_{1b} and the consequent electron-nuclear distances $\bar{r}_i^{exp}$ in the AMP-$MPH^+\cdot$ complex from experimentally obtained values of T_{1p} through the use of eq 7, some accurate estimate of the total concentration of the radical ($[MPH^+\cdot]_0$) and of the correlation time (τ_c) is required. Since these two quantities are dependent on the AMP concentration in the present system, they must be determined for each concentration of 5′-AMP. In the following, experimental methods and the evaluation of these quantities will be described in A and B, respectively, and the application of eq 7 and its consequence will be described in C. In D, we shall discuss probable geometries of the intermolecular complex between 5′-AMP and $MPH^+\cdot$ based on the application of the computer programs I and II. Independent consideration of the mutual orientation of the two molecules will be given in E, based on the expected intermolecular forces.

A. Determination of the Concentration of Free Radical $MPH^+\cdot$. It has been reported that the $MPH^+\cdot$ radical is in an equilibrium with the diamagnetic 5-methylphenazinium cation (MP^+) at pH 6, which is produced by one-electron oxidation of $MPH^+\cdot$ and simultaneous deprotonation at position 10.[23]

$$MPH^+\cdot \rightleftharpoons MP^+ + e^- + H^+ \qquad (10)$$

The integrated area of ESR absorption of $MPH^+\cdot$ was found to increase with the concentration of 5′-AMP indicating that the equilibrium 10 is shifted to the left in the presence of AMP. A quantitative experiment indicates that the fraction of $[MPH^+\cdot]$ in the total ($[MPH^+\cdot] + [MP^+]$) increases from 0.3 in the absence of AMP to a limiting value of 0.7 corresponding to the infinite concentration of AMP. Similar phenomena were reported in the DNA-$MPH^+\cdot$[8] and the DNA-MPCN$H^+\cdot$ (3-cyano-5-methylphenazinium cation radical)[10] systems. The shift of equilibrium would be a result of the preferential complex formation of 5′-AMP with the radical form ($MPH^+\cdot$) rather than with the diamagnetic form (MP^+).

In actual experiments, it is required to estimate the concentration of the free radical in each sample of NMR measurement. For this purpose, the concentration of $MPH^+\cdot$ was conveniently determined from measurement of optical densities directly in the NMR sample tube as described in the Experimental Section. The extinction coefficients were taken to be ϵ_{388} 6100 and ϵ_{446} 11 200 for $MPH^+\cdot$ and ϵ_{388} 26 300 and ϵ_{446} 2300 for MP^+ [23] irrespective of whether it is bound with AMP or not. The actual extinction coefficients of $MPH^+\cdot$ bound with AMP would be different from that in the free form. In order to circumvent this, we made a calibration chart which shows the relation between the "actual" concentration obtained from ESR and the "apparent" one from the optical absorption measurement by using the extinction coefficients above, although the difference between the two methods was less than 10% even at 50 mM AMP.

B. Evaluation of Correlation Times. The correlation times τ_c and τ_e in eq 4 can be expressed by

$$\tau_c = (\tau_r^{-1} + \tau_s^{-1} + \tau_b^{-1})^{-1} \qquad (11)$$

$$\tau_e = (\tau_s^{-1} + \tau_b^{-1})^{-1} \qquad (12)$$

where τ_r is the correlation time characterizing the rotation of the complex, τ_s is the longitudinal relaxation time for the electron spin, and τ_b is the lifetime of the complex as defined earlier. From the line width of a high-resolution ESR spectrum (less than 2×10^{-5} T) measured under the same condition as NMR experiments are done, we estimate the minimum value of τ_e to be 5×10^{-7} s, and therefore $\tau_b \gg 5 \times 10^{-7}$ s. On the other hand, τ_r is of the order of 10^{-10} s as will be shown below so that the contributions of τ_s and τ_b in eq 11 can be neglected, i.e., $\tau_c \simeq \tau_r$.

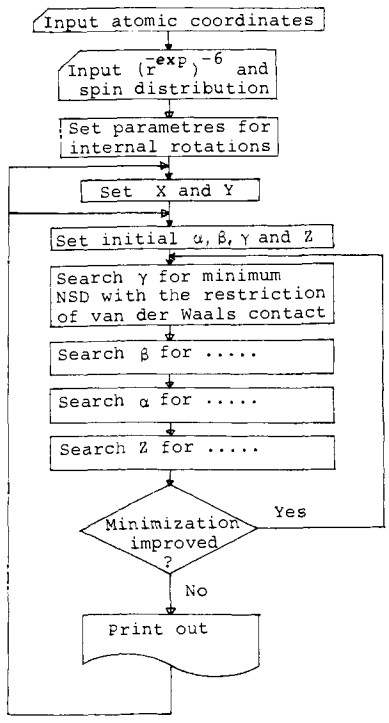

Figure 2. Outline of the flow chart of program I. Conformational parameters are defined in Figure 1.

Direct experimental determination of τ_r for the AMP-$MPH^+\cdot$ complex is difficult. However, according to the Stokes-Einstein equation,[24]

$$\tau_r = 4\pi a^3 \eta / 3kT \qquad (13)$$

we may assume that τ_r is approximately proportional to the volume of the molecular entity. Hence, τ_r of the AMP-$MPH^+\cdot$ complex may be estimated from τ_r of free AMP by mutiplying the ratio of carbon-13 T_1 for free 5′-AMP to that for nicotinamide adenosine dinucleotide (NAD)[16] which would have an equivalent volume to that of the AMP-$MPH^+\cdot$ complex. The ratio was found to be 1.4 at 0.2 M and pH 7, and was assumed to hold at lower concentrations. This assumption is supported by the fact that T_{10}'s of ribose protons of 10 mM NAD are shorter than the corresponding values of 10 mM 5′-AMP by a factor of about 1.4.

The experimental difficulty in obtaining τ_r of free AMP by direct measurement of carbon-13 relaxation times at an extremely low concentration (10 mM) employed in the present work may be avoided by using proton T_{10}'s that can be measured easily down to 5 mM. Table II shows the ratio of τ_r at x mM of 5′-AMP (τ_r^x) to that at 10 mM of 5′-AMP (τ_r^{10}), obtained from T_1 ratios of four ribose protons from the equation

$$\frac{\tau_r^x}{\tau_r^{10}} = \frac{1}{4}\sum_i \frac{(T_{10})_i^{10}}{(T_{10})_i^x} \qquad (i = \text{H1', H3', H4', and H5'}) \qquad (14)$$

This equation is based on the facts that the intramolecular dipolar interaction between protons is a dominant process for the proton longitudinal relaxation, that the extreme narrowing condition is fulfilled, and that the averaged ribose conformation of free 5′-AMP is not significantly changed in the low concentration range of AMP studied.[25,26] By using eq 14, the value of τ_r of the AMP-$MPH^+\cdot$ complex in a 10 mM AMP solution is estimated to be 1.5×10^{-10} s. Small errors introduced in τ_r

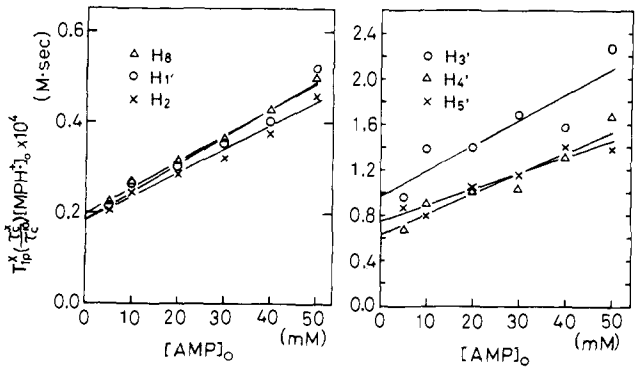

Figure 3. Plots of the normalized relaxation times, $T_{1b}{}^{x}(\tau_r{}^{x}/\tau_r{}^{10})[MPH^{+}\cdot]_0$ against the total concentration of 5′-AMP, $[AMP]_0$ (see eq 7 in the text).

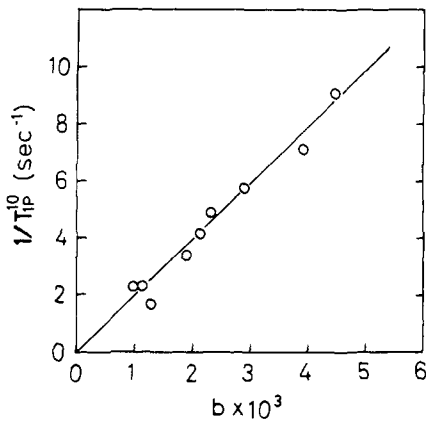

Figure 4. Plots of the enhancement of longitudinal relaxation rate, $(1/T_{1p})$, for H2 of 10 mM 5′-AMP against the fraction of 5′-AMP bound to the $MPH^{+}\cdot$ radical.

Table II. Relative Correlation Times for Various Concentrations of 5′-AMP, Estimated from T_1 of Ribose Protons at 26 °C, pH 6, According to Equation 14 in the Text

x, mM	5	10	20	30	40	50	100[a]
$\tau_r{}^{x}/\tau_r{}^{10}$	0.93	1.00	1.02	1.10	1.19	1.33	1.32

[a] Proton T_1 measurements were done under the same condition as that for the carbon-13 T_1 measurements (28 °C, pH 8) which gives $\tau_r = (1.4 \pm 0.1) \times 10^{-10}$ s.[3]

will little affect electron-nuclear distance ($\bar{r}^{exp}$) calculated therefrom, because of the sixth-power dependence of τ_c on the distance.

C. Elucidation of Association Constant (K), Relaxation Times in the Complex (T_{1b}), and Averaged Electron–Nuclear Distances ($\bar{r}^{exp}$). To estimate the values of $T_{1b}{}^{10}$ and K from eq 7, we measured T_1's of AMP protons at different AMP concentrations in the presence and the absence of $MPH^{+}\cdot$ and plotted them in conformity with eq 7 as shown in Figure 3 (data for H2′ are omitted, because of the enhanced errors at this protons due to the overlap with the HDO signal). The plots make almost straight lines, and $T_{1b}{}^{10}$ and K values obtained from the least-squares fit are given in Table III. The fairly good straight lines of Figure 3 and the reasonable agreement among K values from protons differently located in the 5′-AMP molecule seem to suggest that the complex formation may be represented practically by one unique structure or at least by a family of closely related structures. The averaged value of the association constants between AMP and $MPH^{+}\cdot$ obtained above is 28 M^{-1}. This value is greater than the association constant between 5′-AMP and diamagnetic MP^{+}, which is 20 ± 5 M^{-1} as estimated from an independent measurement of chemical shift of AMP with increasing amount of MP^{+}.[27] The difference between the two association constants is consistent with the fact that the equilibrium 10 shifts to the left with increasing concentration of AMP.

T_1's of protons and phosphorus at fixed concentration of 5′-AMP (10 mM) were also measured for various concentrations of the $MPH^{+}\cdot$ radical. $1/T_{1p}$ in eq 3 is plotted for each nucleus as a function of b calculated by using the averaged binding constant obtained above. For example, $(1/T_{1b}{}^{10})_{H2}$ becomes 1970 ± 160 s^{-1} from the slope in Figure 4 and agrees well with that from eq 7. As shown in Table III, similar agreement exists for the other protons also. Averaged distances $\bar{r}_i{}^{exp}$ between the various nuclei of AMP and the unpaired electron of $MPH^{+}\cdot$ are calculated from $T_{1b}{}^{10}$ values obtained from eq 3 by using eq 4 with $\tau_r = 1.5 \times 10^{-10}$ s, and are listed in Table III.

D. Simulation of the Complex Structure Based on the Comparison of $\bar{r}^{calcd}$ with $\bar{r}^{exp}$. Program I. To examine the probable mutual orientation between 5′-AMP and $MPH^{+}\cdot$ by using program I, which compares $(\bar{r}^{calcd})^{-6}$ with $(\bar{r}^{exp})^{-6}$ for protons from H2 to H4′ as stated in Methods, the glycosidic torsional angle χ and the ribose pucker of 5′-AMP must be fixed. Actually, the preferential conformer of 5′-AMP has been discussed to be syn, anti, or intermediate for the angle χ and 2′-endo or 3′-endo for the ribose pucker.[25,26,28] However, the values of χ are not anything definite and may change in a different environment. In order to fix the parameter χ, we executed program I, varying χ at nine representative positions in the x–y plane (x and $y = -1, 0, 1$ Å). In Figure 5, normalized standard deviations (NSD, eq 9) reached to minimum with respect to z, α, β, and γ at each positional parameter (x, y) are plotted for both 2′-endo and 3′-endo ribose puckers as a function of χ. Since the conformer which shows the lower NSD would be the more probable one, a conformer with 3′-endo anti (χ = 60°, NSD = 4.2%) seems to be most probable. However, 2′-endo anti (χ = 60°, NSD = 6.3%) and 2′-endo syn (χ = 210°, NSD = 6.7%) also seem to have certain probability.

For these three conformers of 5′-AMP, program I was executed again over an extended range in the x–y plane (x and $y = -5, -4, \ldots, 5$ Å) and the results of the calculation for 3′-endo anti are shown in Figure 6. The relative orientations of the adenine ring are shown by arrows, which are defined as B in Figure 1. Three types of arrows show different degrees of agreement between calculated and experimental values of $(\bar{r})^{-6}$ of six protons. Figure 6 does not show the preference of one distinct intermolecular conformation, but a group of "stacked" conformations seems equally allowed. A common finding in these conformers, however, is that the two π planes are nearly parallel with each other with interplane distances between 3.0 and 3.5 Å. As for the results for 2′-endo anti (χ = 60°) and 2′-endo syn (χ = 210°), no orientation which gives NSD less than 6% was found. However, the best agreement was also obtained for a group of stacked conformers similarly as in Figure 6. It is important to note at this stage that no "nonstacked" conformation is allowed for any case. To confirm this, an alternative computation was carried out by varying parameter y instead of z that has been a continuous variable in the previous calculation (Figure 2). It was found that there is no "nonstacked" conformation with NSD less than 11% over any x–z positions (varied in the unit 1 Å) with the angle χ fixed at either syn or anti.

A recent work from our laboratory shows that 5′-AMP in its free state should be as mixture of three major conformers, i.e., anti (3′-endo), syn (2′-endo), and intermediate (2′-endo), and that the dimerization of 5′-AMP, i.e., the stacking interaction between 5′-AMP molecules, shifts the equilibrium exclusively to anti.[26] In the solid state, where intermolecular

Table III. Association Constants (K), Relaxation Rates ($1/T_{1b}$ at 10 mM 5′-AMP), and Electron-Nuclear Distances ($\bar{r}$) for Protons and Phosphorus of 5′-AMP in the AMP-MPH$^+$· Complex in a Neutral D_2O Solution (pH 6, 26 °C)

i	H2	H8	H1′	H2′	H3′	H4′	H5′	P
K, M^{-1} [a]	28.1 ± 5.4	28.9 ± 5.3	34.4 ± 6.0		23.0 ± 9.7	28.9 ± 9.0	18.9 ± 6.4	
$1/T_{1b}^{10}$, s^{-1} [a]	1950 ± 170	1740 ± 90	1610 ± 160		450 ± 100	560 ± 80	710 ± 110	
$1/T_{1b}^{10}$, s^{-1} [b]	1970 ± 160	1790 ± 210	1830 ± 220	680 ± 260	380 ± 170	540 ± 110	580 ± 170	57 ± 30
$\bar{r}_i^{exp}$, Å[c]	4.21 ± 0.06	4.28 ± 0.08	4.27 ± 0.08	5.03 ± 0.38	5.55 ± 0.33	5.23 ± 0.16	5.16 ± 0.21	5.63 ± 0.83
$\bar{r}_i^{calcd}$, Å[d]	4.24	4.32	4.31	6.23	5.59	5.37	4.87	5.55

[a] From Figure 3 by using eq 7. [b] From the plot of eq 3 for each observed nucleus. [c] Experimental values obtained from eq 4 with $\tau_c = 1.5 \times 10^{-10}$ s. [d] Simulated values for the most probable conformation of the complex.

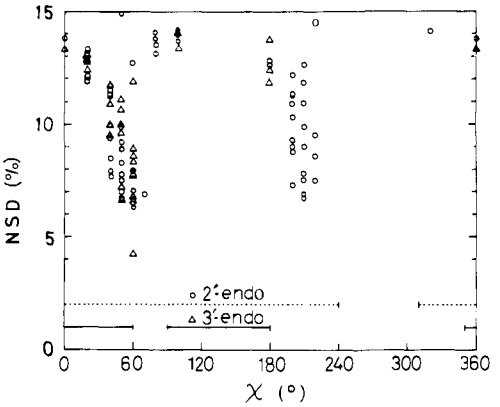

Figure 5. Plots of the minimum values of normalized standard deviations (NSD, eq 9) reached by the program 1, at nine x-y positions (x and y = −1, 0, 1 Å) against the glycosidic torsional angle χ for two ribose conformers of 5′-AMP: ○, 2′-endo; △, 3′-endo. Each position in x-y plane is not specified in this figure. Linear lines represent the allowed regions of χ, based on the repulsion of nonbonding atomic pairs in 5′-AMP of 2′-endo (|· · ·|) and 3′-endo (|—|) conformers.

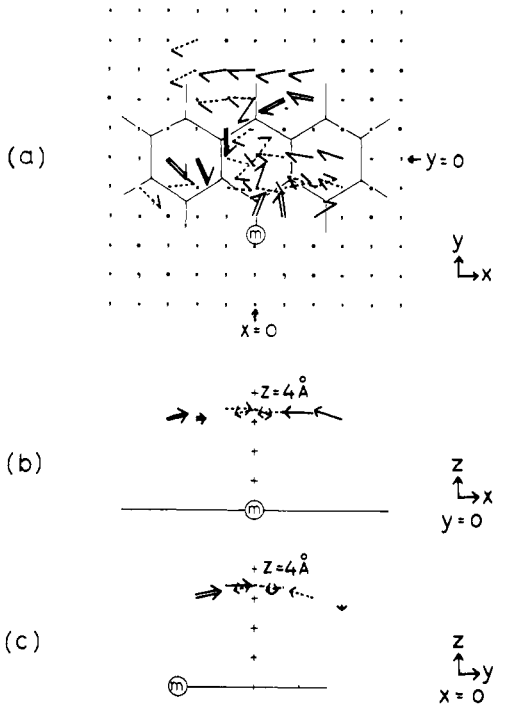

Figure 6. Relative orientations of the adenine ring with respect to the MPH$^+$· plane reached by program 1. The adenine ring is represented by an arrow (defined as B in Figure 2), which gives a minimum value of NSD at each x-y position. Top view (a) and cross sectional views (b) and (c). Types of arrows represent the degree of agreement: ⟸, NSD < 5%; ←, 5% ≤ NSD < 7%; ···, 7% ≤ NSD 9%. (m) represents the methyl group of MPH$^+$·.

interaction is considered to be strong, only the anti conformations are reported for two types of crystals.[29] The monoclinic 5′-AMP takes the 3′-endo anti (χ = 25.7°) whereas the orthorhombic 5′-AMP takes the 2′-endo anti (χ = 72.5°). Two reports on the crystal structure of the complex between a dinucleotide and a drug also indicate that the adenosine moiety of these dinucleotides takes the anti.[30] All these reports seem to suggest that a syn conformer of 5′-AMP which has shown rather lower probability (or greater NSD) may be unlikely in the complex with MPH$^+$·.

Ribose conformation of free 5′-AMP in solution exists as an equilibrium between 2′-endo and 3′-endo with preference for the 2′-endo conformation (63 ± 10%),[25] but at higher concentration of 5′-AMP (and therefore at higher concentration of dimers), the fraction of 3′-endo conformer becomes increasingly greater (≳ 50%).[25,26] Base stacking of dinucleotides and oligonucleotides increases the population of the 3′-endo conformer of the adenyl moiety in solution.[28] The ribose equilibrium also shifts toward the 3′-endo by complexation with tryptamine.[31] The relative stability of the 3′-endo conformer in various complex states of 5′-AMP as stated above seems to be in agreement with the present result that a 3′-endo anti conformer gives the minimum value of NSD in the complex with MPH$^+$·. Although the value of χ for the 3′-endo anti conformer obtained above (60°) is at the edge of the allowed region shown in Figure 5, it is conceivable that a particular intermolecular interaction favors a slightly altered torsional angle. On the other hand, the 2′-endo anti (χ = 60°) conformation with NSD slightly over 6% may not be neglected either. The χ value (60°) is well within a reasonable range predicted by potential energy calculations and is close to the value in the crystal (72.5°).[29] Taking into account these considerations, a reasonable conclusion would be that the conformation of 5′-AMP complexed with MPH$^+$· in solution is the average of two conformers, 3′-endo anti and 2′-endo anti, probably with preference of the former. This conclusion is supported by the fact that the ribose pucker is still in equilibrium between 2′-endo and 3′-endo conformers even in the dimeric state of 5′-AMP[25,26] or in dinucleotides[28] in which states the bases are considered to be stacked with each other.

Program II. To determine the structure of the remaining part of the 5′-AMP in the complex, i.e., angles (ψ,ϕ) about C4′–C5′ and C5′–O5′ bonds, program II was executed on the basis of the result of program I. Only the 3′-endo anti, which is considered to be the major species, was treated by this procedure. By changing angles ψ and ϕ, the deviations of the calculated $(\bar{r})^{-6}$ from the experimental values were minimized for the two C5′ protons and the phosphorus, which had been omitted in the calculation by program I. Since it was difficult to measure T_1's of the two protons separately, we used an averaged value of the two protons in the calculation. No good agreement was found for H5′ and the phosphorus except at around (x,y) = (1, −2) (in Å), which had given NSD = 4.6% by program 1 for the 3′-endo conformer up to the six protons other than the C5′ protons. Program 1 was executed again around this position for the 3′-endo conformer by unit of 0.5

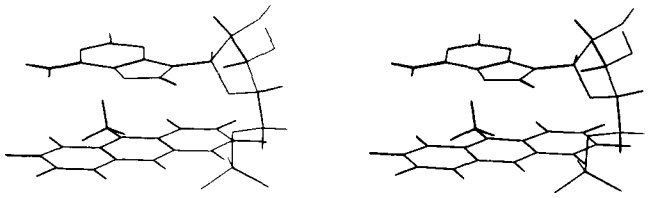

Figure 7. The most probable structure of the complex displayed by program II (stereoscopic view).

Å, and the agreement was checked for all the observed nuclei by using program II. Finally, a complex structure was obtained which showed the best agreement (NSD = 4.9%). As illustrated in Figure 7, this is a typical stacking conformer with an interplane distance (z) of 3.5 Å and the two planes making an angle (α) of only 1.7°, and the exocyclic bond angles (ψ,ϕ) are (43, 182°). The calculated distances ($\bar{r}_i^{\text{calcd}}$) between the electron spin and the various nuclei of 5′-AMP for this structure show good agreement with the experimental ones ($\bar{r}_i^{\text{exp}}$) (see Table III).

This type of complex structure is essentially conserved within about 5% NSD, for the 3′-endo anti conformer, even if we vary τ_c in the range from 1.2×10^{-10} s (z = 2.8 Å, α = 17°) to 1.9×10^{-10} s (z = 3.5 Å, α = 6°), and is considered to be the most representative structure in the 5′-AMP–MPH^+· complex.

The two angles determined by the program II seem to be less accurate for the following two reasons. One of them is the rather low precision of the experimental data for the two protons of C5′ and the phosphorus (see Table III). The other is the possible effect of the local motions about ψ and ϕ on the correlation times τ_c for these nuclei and consequently on the distances between nuclei and electron. In spite of the low accuracy supposed, the values for the angles, (ψ,ϕ) = (43, 182°), are comparable to those in the solid state of 3′-endo 5′-AMP (40.0, 177.2°), and also those of 2′-endo 5′-AMP (62.3, 137.3°).[29] In solution, the gg conformation (ψ,ϕ) = (60, 180°) is reported to be the dominant conformer from NMR coupling analysis for 5′-AMP and dinucleotides.[28] Theoretical calculation also supports the conclusion that this conformer is a preferable one.[32,33]

In this structure of the complex, the oxygen atom of the phosphate group locates only at 3.6 Å from N10 of MPH^+· in the N–H bond direction. If there is any local motion around the phosphate group, the correlation time for phosphorus will become smaller than that of the whole molecule, and the distance between the phosphorus and MPH^+· will become even shorter. The above result strongly suggests the existence of hydrogen bonding between the NH group of MPH^+· and the phosphate group of 5′-AMP. The formation of the hydrogen bond would stabilize the complex structure. The possible presence of the hydrogen bonding between MPH^+· and 5′-AMP could be the reason for the stronger affinity of MPH^+· toward 5′-AMP than that of MP^+ mentioned in C.

E. Independent Check of the Complex Structure from an Energetical Point of View. The structure for the molecular complex shown in Figure 7 has been reached only from the criteria of nuclear magnetic relaxation and the van der Waals exclusive radii. In order to see if the suggested stacking orientation is also a favorable one in the energetical point of view, we have carried out INDO molecular orbital calculations.

Since N-methylphenazinium (MP^+) is known as a substance which forms a molecular complex of charge-transfer type in solid state,[34] we first examine the possibility of this type of interaction in the present system. Represented in Figure 8 is the position of the adenine ring relative to the MPH^+· plane reached by the simulation of the NMR relaxation data as a most probable one (same orientation as in Figure 7). The size of the circle on each atom is drawn proportional to the coefficient of π-atomic orbitals in the lowest unoccupied molecular orbital (LUMO) of MPH^+· and the highest occupied molecular orbital (HOMO) of adenine. (1) There is a good overlap between LUMO and HOMO, indicating a large resonance integral in the charge-transfer state.[35] (2) The calculated energy difference between LUMO and HOMO ($E_{\text{LUMO}} - E_{\text{HOMO}}$ = 1.6 eV) is much smaller than those for usual π–π complexes of charge-transfer type (~4 eV).[35] These two results of the molecular orbital calculation suggest that a relatively large charge-transfer interaction will contribute in the complex formation. Unfortunately, we could not detect a clear charge-transfer band in the optical absorption spectrum.

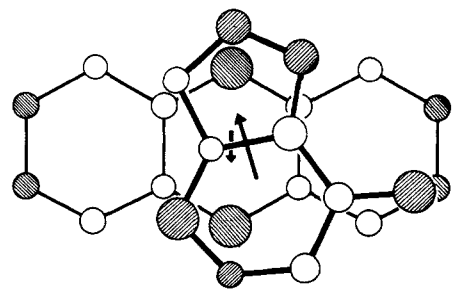

Figure 8. The coefficients of the highest occupied molecular orbital (HOMO) of adenine and of the lowest unoccupied molecular orbital (LUMO) of MPH^+· (represented by the circles) and the permanent dipole moments (represented by arrows), both from INDO calculations. The relative orientation of the adenine ring and MPH^+· is given from the simulation of nuclear relaxation data (same as in Figure 7).

Actual π-electron transmission from the adeneine ring of 5′-AMP to MPH^+· can be estimated by the comparison of $7/6T_{1b}$ with the transverse relaxation rate ($1/T_{2b}$).[36] The result of an independent experiment for ring protons of 5′-AMP indicates that the difference between $7/6T_{1b}$ and $1/T_{2b}$ is so small (~2×10^3 s^{-1}) as to be comparable with experimental errors for T_2 measurements. The above result leads to a rough estimate of the spin transmission of less than about 0.2% of the total spin in the ground state.

Permanent dipole moments which are also obtained by INDO calculations are indicated in Figure 8 by arrows. The magnitudes of the dipole moments are 2.6 D for adeneine and 0.8 D for MPH^+·. The two permanent dipoles in the complex are directed nearly antiparallel, giving nearly the maximal stability due to the dipole–dipole interaction (≃1 kcal/mol with ϵ 1).[37] The first transition moment of the adenine ring is nearly perpendicular[38] and the transition moments of MPH^+· are either parallel or perpendicular to their permanent dipoles from the symmetrical consideration. The interaction between the induced dipole moment of adenine and that of MPH^+· or the mutual polarization between the two molecules are also nearly maximized in the complex formation like that of Figure 8.[39]

We may conclude that a major structure of the complex obtained from the simulation of NMR relaxation data (Figures 7 and 8) is also favorable from the energetical point of view.

Concluding Remark

The existence of π–π stacking between a nucleotide base and a mutagenic dye is directly verified in the present study. This type of interaction, of course, should have a direct relevance to the proposed intercalation model in the interaction of MPH^+· with DNA.[8] The method described in the present report should be able to apply to dinucleotide or oligonucleotide systems including complementary base-paired structures. Such studies are expected to clarify the mode of interaction of a mutagenic dye with DNA in a more direct fashion.

The method presented here will also serve to clarify structures of molecular complex involving various types of free-radical intermediates. An application to a system with a

free-radical derivative of a carcinogen, benzo[*a*]pyrene, will be reported elsewhere.

Acknowledgments. We are indebted to Professors H. H. Dearman (The University of North Carolina), K. Ishizu (Ehime University), and Y. Takagi (Osaka Education University) for continual interest and valuable suggestions during the course of the work.

References and Notes

(1) C. Giessner-Prettre and B. Pullman, *J. Theor. Biol.*, **27**, 87 (1970).
(2) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect", Academic Press, New York, N.Y., 1971.
(3) K. Akasaka, T. Imoto, S. Shibata, and H. Hatano, *J. Magn. Reson.*, **18**, 328 (1975).
(4) H. Sternlicht, R. G. Shulman, and E. W. Anderson, *J. Chem. Phys.*, **43**, 3133 (1965); C. D. Barry, J. A. Glasel, R. J. P. Williams, and A. V. Xavier, *J. Mol. Biol.*, **84**, 471 (1974).
(5) For example, see S. Lesko, W. Caspary, R. Lorentzen, and P. O. P. Ts'o, *Biochemistry*, **14**, 3978 (1975).
(6) For example, see D. E. Edmondson and G. Tollin, *Biochemistry*, **10**, 113 (1971).
(7) K. Ishizu, H. H. Dearman, M. T. Huang, and J. R. White, *Biochim. Biophys. Acta*, **165**, 283 (1968).
(8) K. Ishizu, H. H. Dearman, M. T. Huang, and J. R. White, *Biochemistry*, **8**, 1238 (1969).
(9) Y. Takagi, G. R. Dixson, and H. H. Dearman, *Biochim. Biophys. Acta*, **272**, 12 (1972).
(10) K. Akasaka and H. H. Dearman, *Biochemistry*, **10**, 178 (1971).
(11) K. Akasaka and H. H. Dearman, *Biochem. Biophys. Res. Commun.*, **35**, 377 (1969).
(12) H. H. Dearman, Y. Nosaka, and K. Akasaka, *Biochem. Biophys. Res. Commun.*, **57**, 1125 (1974).
(13) Y. Takagi, K. Akasaka, T. Imoto, H. Kawai, and K. Ishizu, *Chem. Lett.*, 847 (1972).
(14) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 2686 (1964).
(15) I. Solomon, *Phys. Rev.*, **99**, 559 (1955); N. Bloembergen, *J. Chem. Phys.*, **27**, 572, 595 (1957).
(16) W. D. Hamill, Jr., R. J. Pugmire, and D. M. Grant, *J. Am. Chem. Soc.*, **96**, 2885 (1974).
(17) A. V. Lakshminarayanan and V. Sasisekharan, *Biochim. Biophys. Acta*, **204**, 49 (1970).
(18) C. J. Fritchie, Jr., *Acta Crystallogr.*, **20**, 892 (1966).
(19) A more rigorous approach would be to express r^{-6} in terms of the dipolar field tensor from the delocalized unpaired electron as a whole, rather than to express it in terms of r^{-6} contribution from each position of the unpaired electron.
(20) J. A. Pople and D. L. Beveridge, "Approximate Molecular Orbital Theory", McGraw-Hill, New York, N.Y., 1970.
(21) W. C. Hamilton, *Acta Crystallogr.*, **18**, 502 (1965).
(22) A. I. Kitaygorodsky, *Acta Crystallogr., Sect. B*, **27**, 867 (1971); D. E. Williams, *J. Chem. Phys.*, **45**, 3770 (1966).
(23) W. S. Zaugg, *J. Biol. Chem.*, **239**, 3964 (1964).
(24) P. Debye, "Polar Molecules", Dover Press, New York, N.Y., 1945.
(25) F. E. Evans and R. H. Sarma, *J. Biol. Chem.*, **249**, 4754 (1974).
(26) T. Imoto, S. Shibata, K. Akasaka, and H. Hatano, *Biopolymers*, in press.
(27) Y. Nosaka, K. Akasaka, and H. Hatano, unpublished result.
(28) F. E. Evans and R. H. Sarma, *Nature (London)*, **263**, 567 (1976); C.-H. Lee, F. S. Ezra, N. S. Kondo, R. H. Sarma, and S. S. Danyluk, *Biochemistry*, **15**, 3627 (1976).
(29) J. Kraut and L. H. Jensen, *Acta Crystallogr.*, **16**, 79 (1963); S. Neidle, W. Kuehlbrandt, and A. Achari, *Acta Crystallogr., Sect. B*, **32**, 1850 (1976).
(30) C. C. Tsai, S. C. Jain, and H. Sobell, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 628 (1975); N. C. Seeman, R. O. Day, and A. Rich, *Nature (London)*, **253**, 324 (1975).
(31) K. G. Wagner and R. Lawaczeck, *J. Magn. Reson.*, **8**, 164 (1972).
(32) N. Yathindra and M. Sundaralingam, *Biopolymers*, **12**, 297 (1973); R. H. Sarma, C.-H. Lee, F. E. Evans, N. Yathindra, and M. Sundaralingam, *J. Am. Chem. Soc.*, **96**, 7337 (1974).
(33) C. Altona and M. Sundaralingam, *J. Am. Chem. Soc.*, **94**, 8205 (1972).
(34) M. A. Butler and F. Wuld, *Phys. Rev. B*, **12**, 4708 (1975).
(35) R. S. Mulliken and W. B. Person, "Molecular Complexes", Wiley-Interscience, New York, N.Y., 1969.
(36) W. G. Espersen and R. B. Martin, *J. Am. Chem. Soc.*, **98**, 40 (1976).
(37) H. De Voe and I. Tinoco, Jr., *J. Mol. Biol.*, **4**, 500 (1962).
(38) W. Hug and I. Tinoco, Jr., *J. Am. Chem. Soc.*, **95**, 2803 (1973).
(39) P. Claverie, "Molecular Associations in Biology", B. Pullman, Ed., Academic Press, New York, N.Y., 1969, p 115.

A Stopped-Flow Pulse Fourier Transform Nuclear Magnetic Resonance Investigation of the Rates of Chlorination of Metal Acetylacetonates by *N*-Chlorosuccinimide

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Abstract: The rates of chlorination by *N*-chlorosuccinimide at the C3 position of pentane-2,4-dionate ion ($acacH^-$) chelated to Co^{III}, Rh^{III}, Ir^{III}, Al^{III}, and Ga^{III} (M^{III}), in $[M^{III}(acacH)_3]$ and in $[Be(acacH)_2]$, have been measured in a range of solvents at 297 K by stopped-flow pulse FT ^1H NMR. The solvent dependence of the rates and the absence of free radicals in most cases, and of hydrogen isotope effects in the reaction of $[Co^{III}(acacD)_3]$, support an S_E2 mechanism. However, CIDNP-enhanced resonances from intermediates indicate a minor free-radical pathway for the chlorination of the Co^{III} complex in chloroform. The S_E2 reaction rates are not strongly dependent upon which trivalent metal ion is chelated, although the rate for the Be^{II} complex is substantially less than for most of the trivalent metal complexes. The rates can all be rationalized in terms of ligand binding energies and solvent accessibility of the complexes. Substitution by Cl and NO_2 groups (X) at the C3 position of one of the other chelate rings in $[M(acacX)_2(acacH)]$ or $[M(acacX)(acacH)_2]$ slows the chlorination rates of the remaining unsubstituted ligands by factors of between 2 and 12, in a manner analogous to substituent effects in electrophilic aromatic substitution. The presence of a methyl group at the C3 position, however, leads to chlorination at the 3-methylpentane-2,4-dionato ring rather than at the unmethylated pentane-2,4-dionato rings, followed by other side reactions. The corresponding bromination rates, using *N*-bromosuccinimide, are at least 10^6 times as fast as the rates of chlorination, and are too fast to measure by stopped-flow FT NMR even for the least reactive complex investigated, $[Rh(acacCl)_2(acacH)]$.

The mechanisms of the reactions of the coordinated pentane-2,4-dionato ligands in $[M(acacH)_3]$ (M = trivalent metal ion; $[acacX]^- = CH_3COCXC(O^-)CH_3$) have not been extensively studied, although the pseudoaromatic character of the six-membered chelate rings is well documented.[1] Replacement of the methine protons by a wide range of substituents (X) to give $[M(acacX)_3]$ can be achieved usually by the use of electrophilic reagents such as *N*-halogenosuccinimide

0002-7863/78/1500-0713$01.00/0