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ASPECTS OF ENZYME MECHANISMS STUDIED BY NUCLEAR SPIN RELAXATION INDUCED BY PARAMAGNETIC PROBES*

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I. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is unique among spectroscopic methods, sharing with X-ray crystallography the ability to yield experimental parameters concerning individual atoms within a molecule. Information relevant to electronic as well as geometric structure and to molecular motion in solution can be provided by NMR spectroscopy. Biochemical applications of NMR were reviewed in 1964 (1) and were well illustrated in published symposia (2,3). Valuable recent reviews covering the entire field of NMR (4) and its biological applications (5,6) and more particularly ion-solvent interactions (7)

are available. The scope of the present review is more restricted. Its purpose is to examine in detail the use of two NMR parameters that have proved themselves uniquely useful for the study of some enzyme mechanisms: the longitudinal and transverse relaxation rates of nuclear spins of solvent and substrate molecules in the neighborhood of paramagnetic probes. The interpretation of these parameters for individual enzyme systems has been reviewed for kinases (8) and for pyruvate carboxylase (9). The usefulness of relaxation rates due to diamagnetic interactions in the investigation of association of small molecules with macromolecules has been reviewed by Jardetzky (10).

In this review we shall indicate the type of information relevant to enzyme function that has been and can potentially be obtained from measurements of nuclear spin relaxation rates due to paramagnetic probes. These measurements include relaxation rates of the protons of water and/or nuclei of substrates in binary, ternary, and quaternary complexes of enzymes, probes, and substrates or inhibitors in solution. The empirically determined relaxation rates characteristic of each complex can be used as parameters without further interpretation to determine binding constants and the number of binding sites. In some cases kinetic data (ligand exchange rates) and structural information (coordination schemes, number of water ligands, interatomic distances) may be deduced from the magnitude of the relaxation rates. Theoretical analysis of the mechanism of enhancement is not always straightforward but can in favorable cases yield insights into dynamic features, that is, molecular motion at the active site. Where comparisons among physical techniques have been possible, the results obtained by nuclear spin relaxation measurements have shown satisfactory agreement with the results of independent methods for measuring binding (electron spin resonance, ultracentrifugation, chromatography), kinetics (chemical relaxation methods), and molecular distances (X-ray crystallography).

II. Nuclear Spin Relaxation Rates, $1/T_1$ and $1/T_2$

A. PHYSICAL DESCRIPTION OF T_1 AND T_2 PHENOMENA

Before proceeding to a discussion of the special case of nuclear spin relaxation due to paramagnetic species, we consider the phenomena of T_1 and T_2 relaxation in nuclear spin systems in general. The theory of nuclear spin relaxations is presented only qualitatively, stressing the

underlying physical mechanisms; for a more detailed and quantitative treatment the reader is referred to reviews by Jardetzky (10) and Hertz (11).

Nuclei, such as protons, with a spin $I = \frac{1}{2}$, when placed in a static magnetic field H_0, will distribute themselves between two energy levels corresponding to orientations of the spin parallel and antiparallel to the magnetic field. The equilibrium populations of the two energy levels are determined by the energy difference between them ($h\nu = 2\mu H_0$, where μ is the magnetic moment of the nucleus) according to the Boltzmann distribution. If the equilibrium distribution is perturbed (e.g., by changing the magnetic field), a new equilibrium distribution of energy states is approached exponentially with a time constant T_1. The phenomenon may also be described in terms of the component of total magnetization M in the direction of the H_0 field, M_z, approaching its equilibrium value M_0 exponentially, and similarly the component of magnetization in the xy-plane, M_{xy}, approaching 0. The time constants of the two processes are T_1 and T_2, respectively, the longitudinal and transverse relaxation times.

The relaxation phenomena T_1 and T_2 may be visualized in the following way. At equilibrium in the H_0 field the nuclear magnetic dipoles are aligned either with the field (in the $+z$-direction) or against the field (in the $-z$-direction) with more aligned in the $+z$-direction; the vector sum of the magnetic moments of the nuclear spins, that is, the total magnetization, is therefore aligned in the $+z$-direction. Superposition of a strong radio-frequency field in the y-direction for a short time flips the total magnetization into the xy-plane. When the radio-frequency field is removed, the individual dipoles begin to precess about the z-direction. Since the precession frequencies of the individual dipoles are different [$h\nu = 2\mu(H_0 + H_{\text{internal}})$], the precessing dipoles dephase in the xy-plane. As a result, the precessing total magnetization decreases exponentially with time, and the time constant of this process is T_2. Simultaneously with the dephasing process in the xy-plane the dipoles are flipping back to their equilibrium position in the direction of the z-axis by interaction with the "the lattice" as explained below, that is, the T_1 relaxation. Such a removal of the dipole from the xy-plane also decreases the total magnetization in the xy-plane, and thus every T_1 relaxation also contributes to T_2 relaxation. Consequently T_2 is shorter than or equal to T_1.

The T_1 relaxation is effected by the magnetic interaction of a nucleus

with the fluctuating magnetic fields of surrounding magnetic dipoles referred to as the lattice, and hence T_1 is also known as the spin-lattice relaxation time. The magnetic field experienced by any one nucleus due to the random thermal motion of the surrounding molecules containing magnetic dipoles will fluctuate with a frequency spectrum that corresponds to the molecular motion. Only the component of the frequency spectrum that is equal to the resonance frequency, the Larmor frequency ν_o, will be effective in energy exchange that leads to thermal equilibrium, that is, T_1 relaxation. In liquids and solutions the characteristic frequencies of thermal motion are on the order of 10^{11} Hz, much greater than the usual NMR frequencies of 10^7 to 10^8 Hz. Consequently the component of the frequency spectrum from molecular motion that can induce T_1 relaxation is small, and the T_1 relaxation process is slow, about 3 sec for the protons of water. As the molecular motion becomes slower because of lower temperature or molecular size, the intensity of the fluctuation of the magnetic field at the Larmor frequency increases, reaches a maximum, and then decreases. Thus T_1 passes through a minimum value as the molecular motion becomes slower (12). The effect of molecular motion is expressed in the theory by a correlation time τ_c, characteristic of the time of rotation of a molecule or of the time of its translation into a neighboring position.

It should be pointed out that the magnitude of the component of the frequency spectrum at ν_0 and consequently T_1 depends not only on molecular motion (τ_c) but also on the magnitude of magnetic moments in the surrounding nuclei and on intermolecular and intramolecular interactions (e.g., the mean approach distances of the nuclei concerned). The factors contributing to relaxation have been discussed elsewhere in detail (10–12).

As already pointed out, all mechanisms leading to T_1 relaxation also lead to T_2 relaxation. Additional mechanisms of T_2 relaxation arise in solids or in solutions of macromolecules where tumbling is not sufficiently rapid to average out the effect of neighboring spins. In an ensemble of nuclei moving slowly in relation to each other the magnetic field at various nuclei will differ due to neighboring spins, and each nucleus will precess at a different frequency. The range of frequencies is $\Delta\nu$, and the precessing nuclei will be out of phase in a time $\frac{1}{2}\Delta\nu$. More detailed discussions of T_2 mechanisms in interactions between nuclei may be found in texts and reviews (10–12).

B. EFFECTS OF PARAMAGNETIC IONS

Since the magnitude of the magnetic moment of the unpaired electron is about 10^3 times that of a nucleus, the randomly fluctuating magnetic field due to a paramagnetic ion in solution usually dominates the spin relaxation of nuclei in its neighborhood. Thus the introduction of a paramagnetic ion may amplify the nuclear relaxation effect sufficiently for effects to be readily detectable at concentrations of the order of 0.01 mM of the paramagnetic species [e.g., Mn(II)]. Simplification in interpretation is also achieved since the electron–nucleus interaction dominates the relaxation process, and the many nucleus–nucleus interactions may be neglected. However, interpretation is not always straightforward, particularly in complexes with macromolecules, as indicated in the discussion of the theory below. For example, the specific rate process that dominates the nuclear relaxation of water protons in an EMS complex (where E is an enzyme, M is a paramagnetic metal ion, and S is a substrate) can, and often does, change not only when E, M, or S is changed but, for a given EMS, also changes when the temperature is varied.

Essentially there are two types of electron–nucleus interaction that contribute to the nuclear relaxations T_1 and T_2 (see equations 6 and 7 below): a dipolar interaction that depends on the ion-nucleus distance and a scalar (i.e., spin–spin) interaction that depends on the electron-spin density at the nucleus. The correlation time τ_c characterizes the rate process that modulates the dipolar interaction and is given by

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_M} \tag{1}$$

where τ_r is the correlation time characteristic of the rotational motion of the internuclear ion–nucleus radius vector, τ_s is the electron spin relaxation time, and τ_M is the residence time of the nuclear species in the first coordination sphere of the paramagnetic ion ($1/\tau_M$ is the ligand exchange rate between the bound and unbound form). The correlation time is determined by the fastest rate process; that is, whichever time is shortest—τ_r, τ_s, or τ_M. In the aquo complexes of paramagnetic ions of the first transition series elements, where τ_r is of the order of 10^{-11} sec, τ_M is several orders of magnitude longer; τ_s is longer than τ_r for Cu(II), Mn(II), and Cr(III); τ_s is of the same order or shorter than τ_r for most of the other paramagnetic ions (11,13).

The correlation time τ_e for the scalar interaction that is transmitted through chemical bonds rather than through space is given by

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_M} \tag{2}$$

Under most conditions τ_s is shorter than τ_M; at high temperatures for some ions τ_M may become shorter than τ_s, for example, the aquo complex of Mn(II) at temperatures above 80° (12).

C. EFFECT OF CHEMICAL EXCHANGE

If the rate of chemical exchange, $1/\tau_M$, of the nucleus under observation (e.g., the protons of water) between the first coordination sphere of the metal ion and the bulk solvent is fast compared with the relaxation rate in the first coordination sphere, $1/T_{1M}$, then the paramagnetic contribution to the relaxation rate, $1/T_{1p}$, is the weighted average of the relaxation rates in the coordination sphere and the bulk solvent; that is,

$$\frac{1}{T_{1p}} = \frac{1}{T_{1(\text{obs})}} - \frac{1}{T_{1(\text{o})}} = \frac{pq}{T_{1M}} \tag{3}$$

where $T_{1(\text{o})}$ is the relaxation time in the absence of the paramagnetic ion, p is the ratio of the concentration of the paramagnetic ion to the concentration of the ligand, and q is the number of ligands in the coordination sphere; that is, pq is the mole fraction of ligands in the coordination sphere. Although the condition of fast exchange holds for a paramagnetic ion such as Mn(II) free in aqueous solution, it need not hold for the same ion in macromolecular complexes (14). The more general equation for $1/T_{1p}$ (15,16), which takes into account the residence time τ_M of a nucleus in the first coordination sphere, is

$$\frac{1}{T_{1p}} = \frac{pq}{\tau_M + T_{1M}} + \frac{1}{T_{1(\text{os})}} \tag{4}$$

For manganous aquocation, but not for all paramagnetic systems, a similar equation holds for $1/T_{2p}$ (15). The additional term $1/T_{1(\text{os})}$ represents the outer sphere contribution to the relaxation rate, the dipolar interaction of a paramagnetic center with all the nuclei beyond the first coordination sphere.

III. Enhancement of Relaxation Rates in Paramagnetic Macromolecular Systems

A. DEFINITION OF ENHANCEMENT

On binding a paramagnetic ion or free radical to a macromolecule, its effect on the longitudinal relaxation rate $1/T_1$ may be enhanced, particularly for those paramagnetic species, such as manganous ion, where τ_c, the rotational correlation time in the aquocation, is determined by τ_r (cf. equation 1). The enhancement phenomenon was first observed for water protons with some metal complexes of DNA by Eisinger, Shulman, and Szymanski (13). A similar enhancement phenomenon was observed in binary and ternary complexes of proteins with Mn(II) and substrates by Cohn and Leigh (17). Eisinger et al. (13) defined an enhancement factor ϵ, which is the ratio of the relaxation rates in the presence and in the absence of a macromolecule:

$$\epsilon_1 = \frac{1/T_{1p}^*}{1/T_{1p}} = \frac{1/T_1^*{}_{(obs)} - 1/T_1^*{}_{(o)}}{1/T_{1(obs)} - 1/T_{1(o)}} \tag{5}$$

where the asterisk indicates the presence of a macromolecule; $1/T_{1p}$ is the contribution of the paramagnetic ion to the relaxation rate; $1/T_{1(o)}$ is the relaxation rate in the absence of the paramagnetic ion. A similar enhancement factor, ϵ_2, was defined for transverse relaxation rates.

B. THEORY OF ENHANCEMENT

In macromolecular systems tumbling motions and rotational motions are slow compared with those in aquo complexes of paramagnetic ions. Hence an enhancement of the relaxation rate relative to the aquocation may be anticipated for those paramagnetic species, such as $Mn(H_2O)_6^{2+}$, in which τ_c in the aquocation is determined by τ_r, the rotational correlation time (see equation 1). The correlation time in the enhanced system may be τ_r or, if the rotational motion is too slow, τ_s or τ_M may become the relevant correlation time. In any case the new τ_c will be longer than that of the aquocation. Thus T_{1M} will have decreased, and consequently the rate of chemical exchange ($1/\tau_M$) may now be rate limiting for the relaxation even though its magnitude has not changed (cf. equation 4). Because of the slower motion in macromolecular systems and the consequent faster relaxation rates in the coordination sphere of the metal ion, chemical exchange rates that would be fast in

relation to relaxation rates in the hydration sphere of simple aquocations become slow in relation to $1/T_{1M}$ or $1/T_{2M}$ in the macromolecular complex. Thus it becomes possible to detect chemical exchange rates that are too fast to play a role in relaxation in simpler systems. An analogous phenomenon is observed in dielectric dispersion (18).

In order to interpret the observed relaxation rates in terms of molecular parameters of the system it is necessary to examine the theory of the paramagnetic contribution to nuclear relaxation in the coordination sphere of the metal ion. It is assumed that the theoretical treatment that has been worked out for aquo complexes of paramagnetic ions (19,20) also applies to macromolecular complexes. In aqueous solutions the paramagnetic contributions to the T_1 and T_2 relaxation times of protons within the first hydration sphere of a paramagnetic ion, T_{1M} and T_{2M}, are given by the Solomon–Bloembergen equations:

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2}+\frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{2}{3}\frac{S(S+1)\mathrm{A}^2}{\hbar^2}\left(\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (6)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left(4\tau_c+\frac{3\tau_c}{1+\omega_I^2\tau_c^2}+\frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{1}{3}\frac{S(S+1)\mathrm{A}^2}{\hbar^2}\left(\tau_e+\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (7)$$

where S is the electron spin quantum number; γ_I is the nuclear magnetogyric ratio; r, the ion–proton internuclear distance; g, electronic "g" factor; β, the Bohr magneton; ω_I and ω_s, the Larmor angular precession frequency for the nuclear and electron spins, respectively; and A, the hyperfine coupling constant. In equations 6 and 7 the first term represents the dipolar contribution and the second term the scalar contribution to the relaxation rates. For all the cases discussed in this review the value of τ_e is sufficiently large so that $\omega_s^2\tau_e^2 >> 1$, and therefore the scalar contribution to T_{1M} is negligible. However, by the same token, the scalar contribution to T_{2M} may be large. The term $\omega_s\tau_c$ for the water protons in $Mn(H_2O)_6^{2+}$ is approximately 1 at a frequency of approximately 40 MHz, but in the enhanced complexes

$\epsilon \sim 10$, then $\omega_s^2\tau_c^2 >> 1$, and the Solomon–Bloembergen equations simplify to

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \tag{8}$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\tau_e \tag{9}$$

C. DETERMINATION OF MOLECULAR PARAMETERS FROM ENHANCED RELAXATION RATES

In attempting to extract molecular parameters of the macromolecular complex it is simpler to consider the relaxation rate of the macromolecular complex rather than ϵ. The observed value of the longitudinal relaxation rate (equation 4) is a function of the concentration of the paramagnetic species and the following variables: q, the number of ligands containing the nucleus under observation; τ_M, the residence time of the ligand; and T_{1M}, the relaxation time within the first coordination sphere. The latter in turn (equation 8) is a function of r, the distance between ion and nucleus and of τ_c, the correlation time, which may be dominated by τ_r, τ_s, or τ_M (equation 1). In order to interpret the structure and dynamic properties of the complexes one would like to be able to determine the values, or at least limits, of q, r, τ_M, τ_r, and τ_s. The value of q from the relaxation rate of water indicates how many water ligands on a paramagnetic ion have been replaced by the enzyme in a binary complex and by enzyme and substrate in a ternary complex. The distance r limits the possible structure of the complexes; $1/\tau_M$ gives the rate of ligand exchange in the complexes; and τ_r is a measure of the immobilization of the ligand in the coordination sphere of the metal ion.

To disentangle the values of the different parameters from T_{1p} and T_{2p} values there are two variables at our disposal—namely, temperature and frequency. A few generalities may be formulated. If any two of the rate processes represented by T_{1M}, τ_M, τ_s, and τ_r are of the same order of magnitude, the analysis becomes difficult. If the temperature range of investigation is limited by structural changes (e.g., denaturation of protein), difficulties in interpretation are again encountered.

Let us first consider the consequences if τ_M dominates the relaxation rates $1/T_{1p}$ and $1/T_{2p}$. First, if τ_M dominates T_{1p}, $\tau_M > T_{1M}$. Since $T_{2M} \leq T_{1M}$, τ_M must also dominate T_{2p} and consequently $1/T_{1p} \cong 1/T_{2p}$. Second, since τ_M decreases with increasing temperature, $1/T_{1p}$ and $1/T_{2p}$ must increase with increasing temperature. Third, since τ_M is independent of frequency, $1/T_{1p}$ and $1/T_{2p}$ will be independent of frequency. Conversely, if the observed relaxation rates decrease with increasing temperature, then T_{1M} and T_{2M} or outer sphere relaxation determine $1/T_{1p}$ and $1/T_{2p}$. Furthermore, if the observed relaxation rates also depend on frequency, rate processes other than chemical exchange make a significant contribution to T_{1p}. These relationships are tabulated in Table I.

1. Frequency Dependence

If $1/T_{1p}$ is determined by T_{1M} rather than τ_M, it will exhibit a frequency dependence for some values of τ_c. From equation 6, when τ_c is

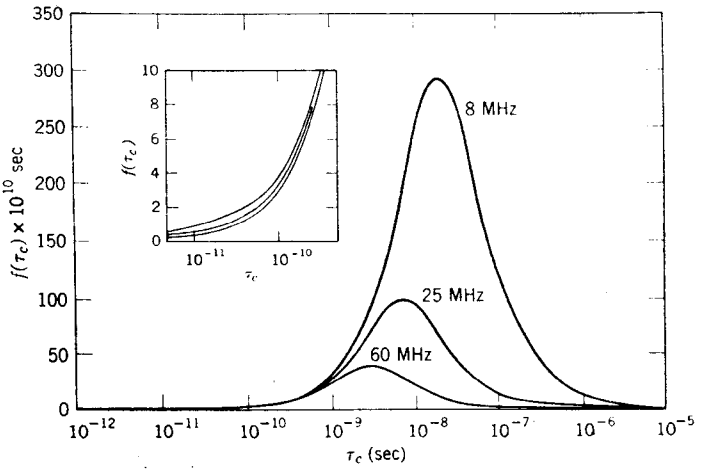

Fig. 1. Frequency dependence of the dipolar term in the Solomon–Bloembergen equation for T_1. $f(\tau_c)$ is plotted as a function of τ_c at 8, 25, and 60 MHz, where

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I{}^2\tau_c{}^2} + \frac{7\tau_c}{1 + \omega_s{}^2\tau_c{}^2}$$

At the maxima, $\omega_I{}^2\tau_c{}^2 = 1$. The insert is enlarged to show the region of τ_c values where changes in ω_s become significant.

TABLE I

Consequences of Domination of $1/T_{1p}$ by a Given Rate Process

Dominant process	τ_c	$d(1/T_{1p})/dT$		$d(1/T_{1p})/d\omega$	T_{1p}/T_{2p}
τ_M		+		0	1[a]
		$\omega_I^2\tau_c^2 < 1$	$\omega_I^2\tau_c^2 > 1$		
T_{1M}	τ_r	−	+	0[b] −	≥1
	τ_s	+ −	+	0[b] − +[c]	>1
	τ_M	−	+	0[b] −	>1
$T_{1(os)}$[d]	τ_d	−		0 0[b]	1
	τ_s	+ −		− +[c]	7/6

[a] If the chemical shift $\Delta\omega$ between the bound and unbound forms is small in comparison with $1/T_{2M}$.

[b] Region where $\omega_s^2\tau_s^2$ is much greater than 1 and $\omega_I^2\tau_c^2$ is much less than 1.

[c] Can occur in the region where τ_s is a function of ω.

[d] The correlation time for outer sphere relaxation is either τ_s or τ_d; the latter is a diffusion time of the order of 10^{-12} sec and is probably the correlation time for small Mn complexes (16).

in the range of 10^{-11} sec, there is a small dispersion due to the ω_s term; when $\tau_c > 10^{-9}$ sec, there is a large dispersion due to the ω_I term. These relationships are illustrated in Figure 1, where the terms in τ_c from the dipolar term of equation 6 [i.e., $f(\tau_c)$] have been plotted as a function of τ_c at three different frequencies. It follows from Figure 1 that at each frequency, if the dipolar term only is important, there is a maximum value theoretically possible for the enhancement factor ϵ_1 where $\omega_I^2\tau_c^2 = 1$, and that maximum increases with decreasing frequency.

The lower the operating frequency, the lower the τ_c value at which a frequency dependence manifests itself.

If $1/T_{1p}$ is a function of frequency, then it should be possible to determine τ_c graphically, as shown by Peacocke, Richards, and Sheard (21); in a plot* of T_1 versus ω_I^2 the ratio of the slope to the intercept is τ_c^2. Once τ_c has been determined in this way, then from equations 3 and 8 q/r^6 may be determined. In the case of the proton relaxation rate of water r is known to be 2.8 Å† (22), and consequently q, the number of water ligands remaining, may be calculated. On the other hand, for a proton in the substrate q is usually 1, and r may be calculated. In principle such calculations should be valid; in the one case analyzed in this way the Mn(II) complex of ribosomal RNA (21), q was calculated to be 3.5. On the other hand, a value of $q = 5$ has been calculated (23) for the Co complex from measurements of chemical shift. A possible resolution of the discrepancy based on rapid rotation of the bound $Mn(H_2O)_5$ complexes about the Mn–phosphate bond (21) leads to values of q that are intermediate between 2 and 5. If this view is correct, it unfortunately vitiates the usefulness of this approach for the precise determination of q.

2. *Temperature Dependence*

Let us first consider the case in which the temperature coefficient of $1/T_{1p}$ is negative; the chemical exchange rate $1/\tau_M$ cannot be the rate limiting process since the rates of chemical reactions have positive temperature coefficients. Therefore $1/T_{1p}$ is determined by T_{1M}‡ (see equation 4), and T_{1M} in turn may be determined by τ_r, τ_s, or τ_M; τ_r and τ_M generally decrease with increasing temperature, but τ_s for Mn(II) complexes may either increase or decrease under different conditions (24).

Let us now consider the second case, in which the temperature coefficient of $1/T_{1p}$ is positive. Three possibilities exist:

* It is assumed that τ_c itself is not a function of frequency; when $\tau_c = \tau_s$, this assumption may not hold, and the plot of T_1 versus ω_I^2 would not be linear.

† It is assumed that the distance from Mn to H of water has not been changed by substituting some of the water ligands.

‡ The possibility that $1/T_{1p}$ is determined by outer sphere relaxation rather than T_{1M} must be considered. It may be ruled out if $T_{1p} > \frac{7}{6}T_{2p}$ and is unlikely if $1/T_{1p}$ is greatly enhanced or the energy of activation is greater than 4 kcal/mole.