Effect of Internal Rotation on Nuclear Magnetic Relaxation Times for Macromolecules†

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ABSTRACT: A previous treatment of the effect of internal rotation on nuclear magnetic resonance relaxation times has been corrected and extended. It is then shown that both diffusional internal rotation and internal rotation by random jumps between three equal potential minima (as for a methyl group) have virtually the same effect on nuclear magnetic resonance relaxation times—these results are illustrated graphically for a wide range of macromolecular sizes, with various choices for the rate of internal rotation and the angle at which the internal rotation occurs. The considerable effect of solution viscosity (as by addition of protein) on the measured relaxation times is demonstrated experimentally. Further experiments with malate and with specifically deuterated malate show that intramolecular dipole–dipole interactions may be expected to dominate in many cases the transverse relaxation time (T_2) for protons. The results from theory and experiment are then combined to yield some procedural optimizations for the extraction of internal rotation information from measured nuclear magnetic resonance times.

Nuclear magnetic resonance (nmr) studies based on the binding, either covalent or labile, of small molecules containing nuclei of spin one-half, such as ^{1}H (Fisher and Jardetzky, 1965; Jardetzky and Wade-Jardetzky, 1965; Burgen *et al.*, 1967; Gerig, 1968; Gerig and Reinheimer, 1970; Schmidt *et al.*, 1969; Sykes *et al.*, 1970; Sykes, 1970; Lanir and Navon, 1971), ^{19}F (Spotswood *et al.*, 1967; Sykes, 1968, 1969; Gerig, 1970), and ^{31}P (Lee and Chan, 1971), or quadrupolar nuclei, such as ^{35}Cl (Stengle and Baldeschwieler, 1966; Marshall, 1970a,b, 1968), ^{25}Mg (Bryant, 1972), ^{23}Na (Jardetzky and Wertz, 1960), ^{39}K (Bryant, 1970), ^{43}Ca (Bryant, 1969), and ^{81}Br (Marshall, 1970a,b), to specific sites on large molecules in dilute aqueous solution have provided a general means for examination of the spatial and chemical organization of the binding site. In particular, molecular rotational motions may be determined from the nmr spectrum and nmr relaxation times. In analyzing these rotational motions, it is often suspected that the attachment of the nucleus of interest to the macromolecular "backbone" is not rigid; for such cases, Wallach (1967) has offered an elegant treatment which predicts the effect of internal rotation of arbitrary rate at an arbitrary angle of attachment of the magnetic nucleus to the macromolecule. While Wallach's treatment is indeed general, his use of an "effective rotational correlation time" is applicable only to macromolecular rotational correlation times, τ_{macro}, which satisfy an "extreme narrowing" condition, $(\omega_0\tau_{macro})^2 \ll 1$. It is becoming increasingly evident that at typical high-resolution nmr frequencies, ω_0, most protein rotational correlation times do not satisfy this condition. In the Theory section, it is shown that if the concept of an effective correlation time is discarded, Wallach's treatment may be extended to describe the effect of internal rotation of nmr relaxation times for macromolecules of any size. In the Discussion the theory is applied to an analysis of experiments involving nmr relaxation times, and a general approach for extracting internal rotation information from such experiments is presented.

Theory

There are four simple models for internal rotation: free rotation, rotational diffusion (random small-angle jumps in angular position), "jump diffusion" (random large-angle jumps in angular position with fixed molecular orientation between jumps), and "free diffusion" (random large-angle jumps with free rotation during the time between jumps). As far as nmr is concerned, the free rotation model will relate only to small molecules in the gas phase at low pressure and will not be discussed here (Oppenheim and Bloom, 1967). For molecules in liquids, each of the remaining models gives a result of similar form (Egelstaff, 1970). Specifically, for quadrupolar nuclei, the dominant contribution to the nmr relaxation times, T_1 and T_2, arises from rotational reorientation of the chemical bond which holds the quadrupolar nucleus to the site of interest; for spin one-half nuclei on small molecules, the contribution from fluctuation of the intramolecular [nuclear-dipole]–[nuclear-dipole] interaction *via* molecular rotation may dominate the relaxation process. For each of these cases, the observed nmr relaxation times will be determined by Fourier transforms of an autocorrelation function for changes in angular position of the molecule or group of atoms. In the presence of diffusional internal rotation (Wallach, 1967), the required autocorrelation function, $\Phi(t)$, takes the form

$$\Phi(t) = {}^{3}/_{4}\sin^4\theta\exp[-(6D_{macro} + 4D_{int})t] + 3\sin^2\theta\cos^2\theta\exp[-(6D_{macro} + D_{int})t] + [(3\cos^2\theta - 1)/2]^2\exp[-6D_{macro}t] \quad (1)$$

while internal rotation by random jumps between three

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potential minima (Wallach, 1967) gives the correlation function

$$\Phi(t) = [{}^3/_4 \sin^4\theta + 3\sin^2\theta\cos^2\theta]\exp[-(6D_{\text{macro}} + (3/\tau_e))t] + [(3\cos^2\theta - 1)/2]^2\exp[-6D_{\text{macro}}t \quad (2)$$

in which θ is the angle between the axis of internal rotation and either the electric field gradient (chemical bond) direction or the internuclear vector, respectively; D_{macro} and D_{int} are the respective diffusion constants for rotational reorientation of the macromolecule as a whole and of the internal rotation; and $(1/\tau_e)$ is the frequency of jumps between discrete angular positions of 0, 120, and 240°.

The correlation functions, eq 1 and 2, vary in time according to either two or three different exponentials, so that there is in general no single well-defined correlation time.[1] However, when molecular rotation is so fast that $(\omega_0/6D_{\text{macro}})^2 \ll 1$, where ω_0 is the Larmor frequency of the resonant nucleus, then the nmr relaxation times T_1 and T_2 depend only on the spectral density

$$J(\omega) = \int_{-\infty}^{\infty}\Phi(t)e^{-i\omega t}\,dt \quad (3)$$

at zero frequency. Now in the *absence* of internal rotation, the relaxation times take the form

$$(1/T_1) = (1/T_2) = (\ )^2J(0) \quad (4)$$

where $(\ )^2$ represents the squared magnitude of the interaction responsible for the relaxation process; in this case, τ_c may be defined correctly by

$$\tau_c \equiv J(0) = (1/6D_{\text{macro}}) \quad (5)$$

Following eq 5 Wallach was led to define an effective correlation time in the presence of internal rotation by

$$\tau_{\text{eff}} \equiv J(0) = {}^3/_4\sin^4\theta\,[1/(6D_{\text{macro}} + 4D_{\text{int}})] + 3\sin^2\theta\cos^2\theta\,[1/(6D_{\text{macro}} + D_{\text{int}})] + [(3\cos^2\theta - 1)/2]^2[1/6D_{\text{macro}}] \quad (6)$$

for a diffusional model of internal rotation. In the limit of very rapid internal rotation, $D_{\text{int}} \gg D_{\text{macro}}$, eq 6 implies that

$$\tau_{\text{eff}} = [(3\cos^2\theta - 1)/2]^2[1/6D_{\text{macro}}] \quad (7)$$

leading to the conclusion that fast internal rotation acts to decrease the rotational correlation time. (Equation 7 follows also from the jump model for internal rotation, when the jump frequency is much larger than D_{macro}.)

However, inspection of eq 1 or 2 quickly reveals that even for very fast internal rotation, when all but the last term in eq 1 or 2 may be neglected, one obtains the correlation function

$$\Phi(t) = [(3\cos^2\theta - 1)/2]^2\exp[-6D_{\text{macro}}t] \quad (8)$$

having the *same* correlation time, $(1/6D_{\text{macro}})$, as if no internal rotation were present. Thus the effect of rapid internal rotation is *not* to reduce the rotational correlation time, but rather to reduce the effective magnitude of the interaction causing relaxation. This distinction can be of importance in analysis of T_1 and T_2 results, as shown later.

Experimental Section

Halide Nuclear Magnetic Resonance. Each sample for nmr measurements contained 0.05 M phosphate, pH 7.0, and either 2.0 M NaCl or 1.0 M NaBr. Some samples also contained 0.2% diisopropylphosphoryl-chymotrypsin (DIP-chymotrypsin). The lower bromide concentration is possible because of the greater nmr sensitivity for ^{81}Br compared to ^{35}Cl. Solution viscosity was varied by addition of sucrose. Relative viscosities (compared to distilled water) were obtained from the time required for a bulb of liquid to discharge through the capillary of an Ostwald viscosimeter, and from corresponding density measurements based on the weight of a 10-ml sample of the same liquid. All viscosities were determined at 23°.

^{35}Cl nmr spectra were obtained at 4.33 MHz, using a Varian HR-60 nmr spectrometer with a V-4311 fixed-frequency radiofrequency unit. The ^{35}Cl nmr signal was modulated at 400 Hz and observed as the first side band, with the use of a P.A.R. Model JB-4 lock-in amplifier to eliminate base-line drift. Chlorine line widths were calibrated from the separation of the side bands from a saturated aqueous NaCl sample. ^{81}Br nmr spectra were detected at 8.0 MHz, using a Varian Model V4210A variable-frequency radiofrequency unit and 4–8-MHz "probe." The ^{81}Br nmr signal was modulated at 3000 Hz, observed as the first side band, and calibrated from the side-band separation for a saturated KBr sample. In this case an external Hewlett-Packard audiooscillator was used in place of the internal oscillator of the lock-in amplifier for better noise limiting. All nmr experiments were conducted at ambient temperature, and each reported line width is the average of at least ten spectra; error bars indicate the mean absolute deviation from an average value.

Proton Nuclear Magnetic Resonance. Proton relaxation times were measured for inhibitors of the catalytic subunit of aspartate transcarbamylase (Schmidt *et al.*, 1969; Sykes *et al.*, 1970). L-Malate and specifically deuterated L-malate (one methylene proton and the α proton deuterated to yield *α-threo-β*-dideuterio-L-malate (Seaver *et al.*, 1972)[2]) were compared for the effect of enzyme on their methylene proton relaxation times. In these experiments T_1 and T_2 were obtained at 100 MHz for carbon-bound protons of the inhibitors by the method of adiabatic half-passage or T_1 in the rotating frame ($T_{1\rho}$) (Sykes, 1969). A Varian HR-100 instrument was used with fully protonated L-malate solutions while a Varian HA-100 was used with deuterated L-malate (benzene in a concentric capillary provided the lock signal). Sufficient radiofrequency power was used to collapse the methylene multiplet of L-malate so that the magnetization decayed with a single time constant $T_{1\rho}$. However, the radiofrequency power level was still low enough that $T_{1\rho}$ equalled T_2 (Schmidt, 1970). T_1 was obtained by progressively varying the time off resonance in the $T_{1\rho}$ experiment (Sykes, 1969).

Solutions for relaxation studies contained 0.1 M L-malate, 0.02 M carbamyl phosphate (one of the enzyme substrates), and varying concentrations of enzyme. The buffer contained 0.01 M imidazole acetate, 0.04 M glycylglycine, 2×10^{-3} M

[1] A correlation time, τ_c, is most generally defined by the condition that $\Phi(t)$ be very small for times, $t \gg \tau_c$.

[2] To be published.

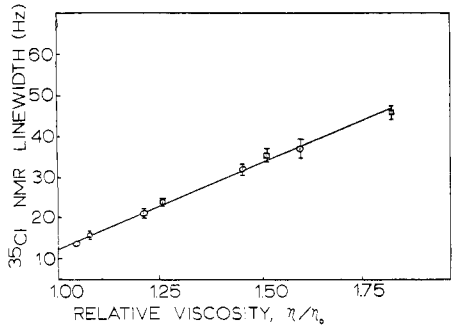

FIGURE 1: Plot of ^{35}Cl nmr line width *vs.* solution relative viscosity, η/η_0; η_0 is the viscosity of distilled water. Viscosity is varied by addition of sucrose. (⊙) 2.0 M NaCl–0.05 M phosphate (pH 7.0). (⊡) 2.0 M NaCl–0.05 M phosphate (pH 7.0)–0.2% DIP-chymotrypsin.

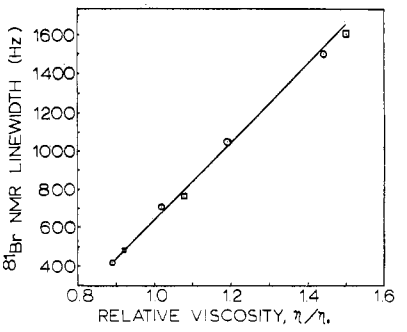

FIGURE 2: Plot of ^{81}Br nmr line width *vs.* solution relative viscosity, η/η_0; η_0 is the viscosity of distilled water. Viscosity is varied by addition of sucrose. (⊙) 1.0 M NaBr–0.05 M phosphate (pH 7.0). (⊡) 1.0 M NaBr–0.05 M phosphate (pH 7.0)–0.2% DIP-chymotrypsin.

2-mercaptoethanol, and 2×10^{-4} M EDTA. All solutions were in D_2O, pH meter reading 7.0.

In proton work, the presence of any paramagnetic contaminants can vitiate any attempts to quantitate measurements of relaxation times, since the paramagnetic species itself contributes to proton relaxation. It is thus advisable to rigorously exclude paramagnetic metal ions from solutions of macromolecules; where feasible, one method is to include enough EDTA in the solution to scavenge metal ions so they are not available to bind to either small or large organic species (Skyes *et al.*, 1970).

Discussion

Various methods for extracting rate information about chemical binding processes and molecular motion from nmr measurements have recently been reviewed (Sykes and Scott, 1972). We will now outline briefly some procedural optimizations for a number of selected experiments, based in part on the results of the Theory section.

Quadrupolar Nuclei. Typical studies of macromolecules using quadrupolar nuclei have often involved the exchange of halide (^{35}Cl, ^{81}Br) nuclei between two sites: (A) free halide (0.5 M in water) and (B) halide bound to a mercury which is located at a site of interest on a macromolecule at considerably lower concentration (10^{-5} M) (Stengle and Baldeschwieler, 1966). The parameter of interest is $1/T_2$(B), and if the chemical exchange rate between sites A and B is of the appropriate magnitude (Marshall, 1970a,b), then $1/T_2$(B) may be determined from measurement of the halide nmr full line width at half-height.

$$\Delta\nu_{obsd} = f_A\Delta\nu_A + f_B\Delta\nu_B \quad (9)$$

$$\Delta\nu = (1/\pi T_2) \quad (10)$$

where f_A and f_B are the respective fractions of halide at A sites or B sites.

Information about internal rotation is obtained as follows. Since the resonant frequency for halide nuclei is much less than for protons (at magnetic fields of, say, 10,000 G), the "extreme narrowing limit," $(\omega_0\tau_{macro})^2 \ll 1$, will be approximately valid even for halide bound to a macromolecule. Thus $T_1 = T_2$, and in particular

$$1/T_2(B) = (2\pi/5)(e^2qQ)^2J(0) \quad (11)$$

in the presence of diffusional internal rotation, where (e^2qQ) is the quadrupole coupling constant for the Hg–Cl bond in the compound, RHgCl. Thus when D_{macro} and (e^2qQ) are known from independent experiments, a reduction in $1/T_2$(obsd), compared to the result expected for halide rigidly bound to the macromolecule, is evidence that internal rotation is present. Previous conclusions from such results in the literature (Wallach, 1967; Marshall, 1968) are still valid, because this conclusion is unaffected by whether the reduction factor (which would be $[(3\cos^2\theta - 1)/2]^2$ for rapid internal rotation) is associated with the correlation time, $(1/6D_{macro})$ (the incorrect view, see Theory), or with the quadrupole interaction magnitude (the correct view).

There is an additional important procedural motion. The ordinary means of conducting the experiment is to vary the concentration of macromolecule, keeping the concentration of free halide constant. Equation 9 would then seem to indicate that when free halide is in large excess, [A] $\gg$ [B], and $1/T_2$(B) might be obtained simply as the slope of a plot $[1/T_2(obsd) - 1/T_2(A)]$ *vs.* f_B, where $1/T_2$(A) is the line width for free halide in the presence of macromolecule. However, the typical macromolecular concentration is often sufficiently large to change the viscosity of the solution appreciably, so that $1/T_2$(A) will generally be larger in the presence than in the absence of protein. The importance of this effect may be seen in Figures 1 and 2, in which the dependence of free halide nmr line width on viscosity is shown, where viscosity was varied by addition of sucrose. The fact that the squares and circles fall on the same straight line indicates that there is no binding of halide to the (inert) DIP-chymotrypsin. The left-most circle and left-most square in each figure correspond to solutions which contained no sucrose at all—the difference in line width between the left-most circle and the left-most square in either plot is thus wholly attributable to the increase in solution viscosity due to addition of protein.

Therefore, in extracting the desired parameter $1/T_2$(B) from the macromolecular concentration dependence of $1/T_2$(obsd), is clearly necessary to include control solutions of free halide having the same viscosity as the sample which contains protein. Finally, the same caveat applies to any experiment in which the viscosity of the solution is affected, as, for example, in attempts to denature a protein by addition of a high concentration of urea.

Margalit (1971) has studied internal rotation in deuterated *tert*-butyl alcohol. However, this molecule is sufficiently small that its rotational correlation time is short enough to satisfy the "extreme narrowing" condition, $(\omega_0/6D_{rot})^2 \ll 1$,

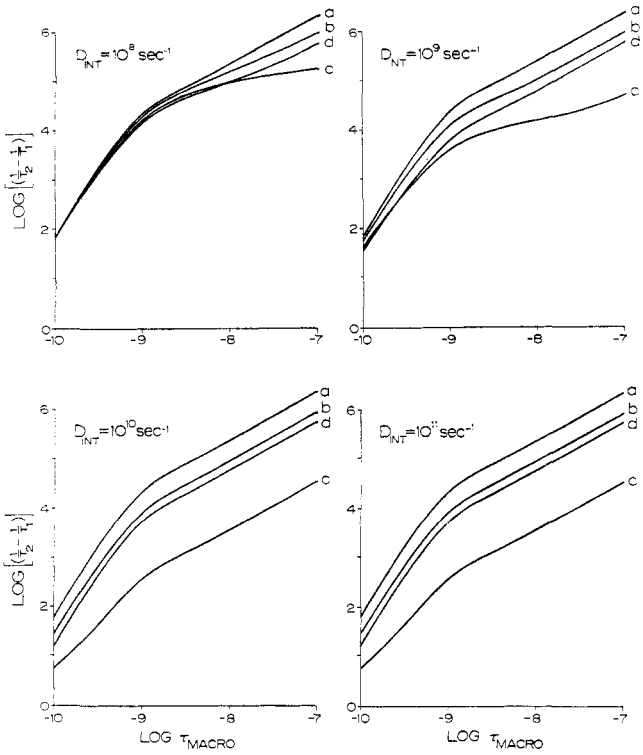

FIGURE 3: Effect of diffusional internal rotation on $1/T_2(B) - 1/T_1(B)$. D_{macro} is the rotational diffusion constant for the macromolecule, D_{int} is the rotational diffusion constant for the internal rotation, θ is the angle at which the internal rotation occurs, and $T_2(B)$ and $T_1(B)$ are the nmr relaxation times for the small molecule when it is attached to the macromolecule. $\omega_0 = (2\pi) \times 10^8$ rads/sec. (a) $\theta = 0°$, (b) $\theta = 30°$, (c) $\theta = 60°$, and (d) $\theta = 90°$. Origin of the y axis is arbitrary.

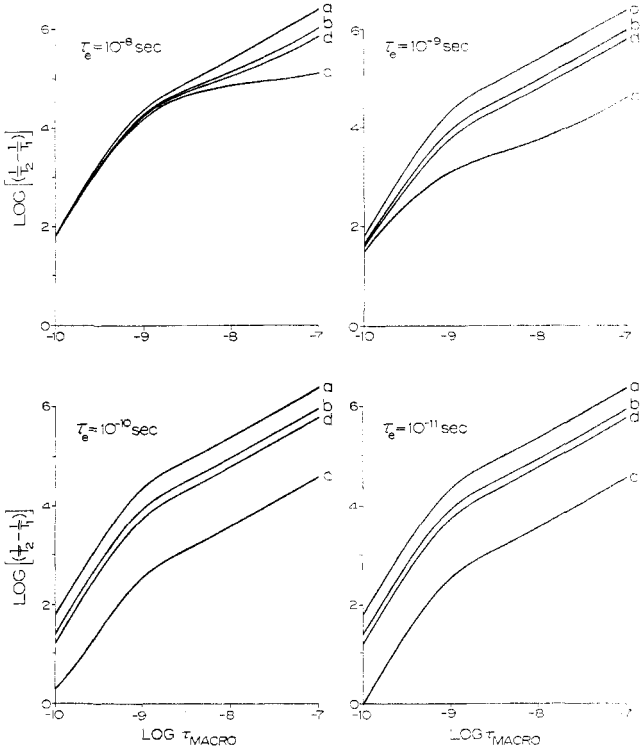

FIGURE 4: Effect on $1/T_2(B) - 1/T_1(B)$ of internal rotation by random jumps between three potential minima. D_{macro} is the rotational diffusion constant for the macromolecule; $1/\tau_e$ is the rate of jumping between three equally likely angular positions having azimuthal angles of 0, 120, and 240°; θ is the polar angle at which internal rotation occurs, and $T_2(B)$ and $T_1(B)$ are the nmr relaxation times for the small molecule when it is attached to the macromolecule. $\omega_0 = 2\pi \times 10^8$ rads/sec. (a) $\theta = 0°$, (b) $\theta = 30°$, (c) $\theta = 60°$, and (d) $\theta = 90°$. Origin of the y axis is arbitrary.

so that the considerations of the present Theory section are not required for successful interpretation of the results.

Binding and Exchange of Small Molecules with Macromolecules. Ordinarily, macromolecular concentration is so low that there is a large excess of free small molecule. Under these conditions, the equations which relate the observed chemical shift, T_1, and T_2 to the "off" rate for binding, the chemical shift difference Δ between free and bound forms of the small molecule, concentrations of the two forms, and the intrinsic T_1 and T_2 for each of the two sites A and B take on especially simple form (Swift and Connick, 1962). Even with this simplification, extraction of the above parameters from the measured quantities is generally possible only when the "off" rate is either "fast" or "slow." Sykes, Schmidt, and Stark have given a procedure (Sykes *et al.*, 1970) for establishing when the off rate is in fact fast or slow, based on experiments at different magnetic fields, followed by experiments at different temperatures. When the off rate is slow, the off rate may be obtained from the concentration dependence of $1/T_2$(obsd). When, on the other hand, the off rate is fast, it is possible to obtain Δ from the concentration dependence of the observed chemical shift, while $(1/T_1)_{bound}$ and $(1/T_2)_{bound}$ may be obtained from the respective concentration dependence of $1/T_1$(obsd) and $1/T_2$(obsd).

$$\frac{1}{T_1(\text{obsd})} = f_A\frac{1}{T_1(A)} + f_B\frac{1}{T_1(B)} \quad (12)$$

$$\frac{1}{T_2(\text{obsd})} = f_A\frac{1}{T_2(A)} + f_B\frac{1}{T_2(B)} \quad (13)$$

As in the case of the halide probe experiments $T_1(B)$ and $T_2(B)$ can be obtained from plot of $1/T_1(\text{obsd}) - 1/T_1(A)$ *vs.* f_B and $1/T_2(\text{obsd}) - 1/T_2(A)$ *vs.* f_B, respectively. This requires, however, that $1/T_1(A)$ and $1/T_2(A)$ be measured under exactly the same conditions as $1/T_1$(obsd) and $1/T_2$(obsd) were determined. One procedure might be to measure $1/T_1(A)$ and $1/T_2(A)$ for the small molecule in the presence of macromolecule which has been modified so that the binding site is blocked. A simpler procedure, since $1/T_1(A) = 1/T_2(A)$ for most nuclei in small molecules, is to determine $(1/T_2(B) - 1/T_1(B))$ from the slope of a plot of $(1/T_2(\text{obsd}) - 1/T_1(\text{obsd}))$ *vs.* f_B. Calculating the data in this way has the distinct advantage of automatically compensating for viscosity effects since $1/T_1(A)$ and $1/T_2(A)$ are affected to the same degree.

To ascertain whether or not internal rotations are affecting the observed relaxation times, an assumption must now be made about the dominant relaxation mechanism for the nuclei in question. In many cases (see below), the relaxation of spin $^1/_2$ nuclei is dominated by intramolecular dipole–dipole interactions both when the small molecule is free in solution and when it is bound to the macromolecule. If this is true for the system of interest, the expected value of $1/T_2(B) - 1/T_1(B)$ in the absence of internal rotation can then be calculated using eq 14 (Sykes *et al.*, 1970)

$$\left(\frac{1}{T_2(B)} - \frac{1}{T_1(B)}\right)_{calcd} = \sum_{ij}\frac{3}{40}\left(\frac{\hbar^2\gamma^4}{r_{ij}^6}\right) + \left[6\tau_{macro} + \frac{6\tau_{macro}}{1+(\omega_0\tau_{macro})^2} + \frac{12\tau_{macro}}{1+4(\omega_0\tau_{macro})^2}\right] \quad (14)$$

the known values of the internuclear distances, and τ_{macro} determined from other measurements (see below). Equation 14 is valid for intramolecular dipole–dipole relaxation only, whereas it might be expected that, in general, both intra- and intermolecular processes could be important in the type of experiment discussed here. Studies have been carried out to assess the relative contributions of the two processes (Schmidt, 1970). In the presence of the catalytic subunit of aspartate transcarbamylase plus carbamyl phosphate, the methylene protons of L-malate were strongly relaxed due to the binding of the small molecule at the active site [$(1/T_2(B)) - (1/T_1(B)) = 270$ sec^{-1}]. Under identical conditions except that, in the L-malate, deuterium was substituted for one methylene proton and the α proton, the relaxation effect was less than 0.17 that with the fully protonated inhibitor ($1/T_2(B) - 1/T_1(B) = 45$ sec^{-1}); because of its small magentogyric ratio, deuterium is much less effective than protons in causing dipole–dipole relaxation. In fact, 0.17 represents an *upper bound* because chemical exchange processes which are independent of dipole–dipole coupling also contribute to the relaxation in both specifically deuterated and fully protonated L-malate (Seaver *et al.*, 1972).[2] Intramolecular processes therefore dominate the methylene proton dipole–dipole relaxation of L-malate. Similar behavior may be expected for methylene and methyl groups of other systems. However, methine and aromatic proton relaxation (Lanir and Navon, 1971), because of increased internuclear distances, may not be dominated by intramolecular processes.

If the calculated value of $1/T_2(B) - 1/T_1(B)$ is appreciably greater than the measured value, then internal rotation may be present (other relaxation mechanisms can only increase the observed value). The next step is to correct the calculated value for obvious fast internal motions ($D_{int} \gg D_{macro}$), such as the internal rotation of a methyl group about its threefold axis, and then again compare the calculated and observed results. If the observed value of $1/T_2(B) - 1/T_1(B)$ is still measurably smaller than the calculated result, then there must be some genuine local flexibility at the bound site. The nature of this internal motion can then be estimated by comparison of the observed results to the plots of $[1/T_2(B) - 1/T_1(B)]/(\ )^2$ presented in Figures 3 and 4 for the various values of τ_{macro} and the parameters characterizing internal rotation. This will in general require the additional assumption that one of the parameters (rate of internal rotation or angle of attachment) is known from a knowledge of the system. Caution must certainly be exercised at this stage.

Solution of eq 14 requires that τ_{macro} be known. Values for τ_{macro} of proteins have been measured with a number of techniques including fluorescence depolarization (Weber, 1953), spin labels (Shimshick and McConnell, 1972), and dielectric dispersion (Takashima, 1969). The treatment presented in the Theory suggests another method. The spin–lattice relaxation rate is given by

$$1/T_1 = (\ )^2[J(\omega_0) + J(^2\omega_0)] \qquad (15)$$

In the limit of *either* fast or slow internal rotation ($D_{int} \gg D_{macro}$ or $D_{int} \ll D_{macro}$) $1/T_1$ is a function of only one correlation time, τ_{macro} (see eq 1 and 3). For this case and assuming intramolecular dipole–dipole relaxation

$$\frac{1}{T_1} = (\ )^2\left[\frac{4\tau_{macro}}{1 + (\omega_0\tau_{macro})^2} + \frac{16\tau_{macro}}{1 + 4(\omega_0\tau_{macro})^2}\right] \qquad (16)$$

where $(\ )^2$ now includes the angular factors for internal rotation, if present (these need not be known, however). If $1/T_1$ is measured as a function of ω_0, the data may be fitted to yield τ_{macro}. Koenig and coworkers (Koenig and Schillinger, 1969; Koenig *et al.*, 1971) have described a method for measuring T_1 of H_2O in protein solutions over the frequency range of 0.01–100 MHz. This corresponds to the region of interest for most anticipated values of τ_{macro}.

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