THEORY OF RELAXATION OF MOBILE WATER PROTONS INDUCED BY PROTEIN NH MOIETIES, WITH APPLICATION TO RAT HEART MUSCLE AND CALF LENS HOMOGENATES

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ABSTRACT Kimmich and co-workers (cf., Winter, F., and R. Kimmich. 1982. *Biochim. Biophys. Acta.* 719:292–298) discovered peaks in the magnetic field-dependent longitudinal relaxation rate ($1/T_1$) of water protons of muscle tissue, cells, and dehydrated protein in the field range 0.5–5 MHz (proton Larmor frequency), and argued that the peaks resulted from cross relaxation associated with quadrupolar splittings of the ^{14}N nuclei of protein NH groups. More recently, analogous peaks were found in homogenates of calf eye lens (Beaulieu, C.F., J. I. Clark, R. D. Brown III, M. Spiller, and S. H. Koenig, 1987. *Abstr. Soc. Magn. Res. Med., 6th, New York.* 598–599), which are essentially concentrated protein solutions, and were measured with sufficient precision to allow resolution of the relaxation spectra into several peaks and the intrinsic linewidths to be determined. Here, we analyze these relaxation spectra, as well as earlier data on rat heart (Koenig, S. H., R. D. Brown III, D. Adams, D. Emerson, and C. G. Harrison. 1984. *Invest. Radiol.* 19:76–81) in some detail, and suggest a specific pathway for the cross relaxation to which we apply the theory of relaxation quantitatively. The view that emerges is that, at fields such that the proton Zeeman energy of the NH protons matches an ^{14}N quadrupolar splitting, relaxation of these protons is by cross relaxation to the ^{14}N nuclei which in turn transfer excess energy to the protein. The correlation time for the NH proton interaction is the T_2 of the ^{14}N nuclei, $\sim 10^{-6}$ s, whereas T_1 of the NH protons is ~ 1.25 ms. At these energy level crossings, the NH protons become relaxation sinks for protons of rapidly exchanging ($\sim 3 \times 10^9$ s^{-1}) water molecules hydrogen bonded to the same backbone carbonyl oxygens as the NH protons. The lifetime of this hydrogen bond ($\sim 3 \times 10^{-10}$ s) then becomes the correlation time for the water proton–NH proton interaction which, though short, is much longer than the analogous correlation time ($\sim 5 \times 10^{-12}$ s) in pure water; the enhanced interaction results in peaks in the field-dependent $1/T_1$ of the solvent protons. There are few data on the lifetime of such bonds, but the results here conform with the recent considerations of Bennett, H. F., R. D. Brown III, S. H. Koenig, and H. M. Swartz. 1987. *Magn. Reson. Med.* 4:93–111, regarding hydrogen bond lifetimes for water molecules bound to macromolecules. The recent precise field-dependent relaxation data, here combined with both a quantitative theory and the fact that the magnitude of the ^{14}N peaks is very concentration sensitive, allow, at least for lens proteins, a study of protein–protein interactions difficult to investigate by other methods.

INTRODUCTION

It has been known for almost two decades that the presence of solute diamagnetic protein influences the magnetic relaxation rates of solvent water protons (Daszkiewicz et al., 1963; Koenig and Schillinger, 1969); these rates become field- and temperature-dependent in a manner that indicates that the water molecules sense the rotational Brownian motion of the protein molecules (Hallenga and Koenig, 1976). Though the molecular mechanisms of solute–solvent interaction are not understood in detail, three classes of interactions can be important: (*a*) pure hydrodynamic effects (Koenig, 1980); (*b*) exchange from a hydration sphere in which water molecules reside with an appropriate lifetime (Koenig and Schillinger, 1969); and (*c*) cross relaxation with nuclei, predominantly protons, of the solute protein (Koenig et al., 1978). The result is a field-dependence of $1/T_1$, the longitudinal relaxation rate, called an NMRD (nuclear magnetic relaxation dispersion) profile, that decreases monotonically with increasing field, with an inflection point at a Larmor frequency related to the inverse of the orientational relaxation time of the protein molecules. The relaxation behavior of water protons in most tissue samples is very similar to this, except that the NMRD profiles suggest a heterogeneous sample, consistent with the presence of a range of protein sizes and conformations (Koenig et al., 1984); indeed, in most ways, tissue behaves much like a heterogeneous macromolecular solution (Koenig and Brown, 1985).

With the advent of nuclear magnetic resonance imaging (MRI), and its increasing utility as a clinical diagnostic

tool, the need to understand the molecular basis of relaxation of water protons has become of increasing import. Most tissues have almost identical water content, and image contrast between different organs depends mainly on tissue-specific relaxation rates, the causes for which remain to be clarified (Koenig and Brown, 1987). In tissue and in cells, a small amount of structure in the 0.5–5 MHz decade of field (expressed in units of proton Lamor frequency) can often be detected superimposed on the underlying monotonic NMRD profile (Winter and Kimmich, 1982*a*, *b*; Koenig et al., 1984). This structure, a series of peaks in $1/T_1$, arises from cross relaxation between protons of mobile water and ^{14}N nuclei of tissue or cellular protein. No such structure had been reported for protein solutions until the recent work of Beaulieu et al. (1987), who demonstrated large ^{14}N peaks in homogenates of calf lens (essentially a protein solution). They attributed the magnitude of these peaks to unusual protein–protein interactions responsible for the short range order found in lens (Delaye and Tardieu, 1983), which would make the majority of the backbone NH moieties accessible to solvent. These data—essentially relaxation spectra—are so rich that it now becomes of interest to clarify the pathway for the cross relaxation and establish that the observations can be accounted for quantitatively by theory, which is the aim of the present work.

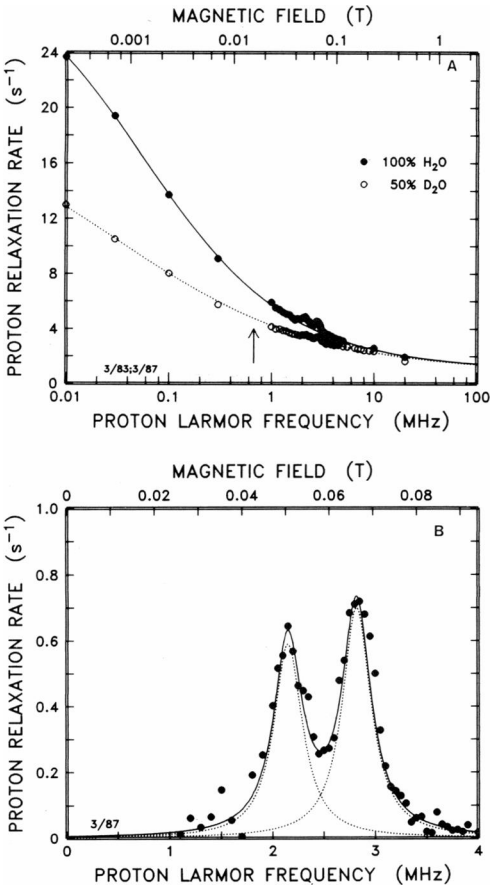

FIGURE 1 (*A*) $1/T_1$ NMRD profiles of rat heart muscle at 10°C before and after partial deuteration. The continuous curves are empirical fits to the underlying monotonic variation of the relaxation rates with field. The arrow indicates where structure in addition to that seen in the 1–5 MHz region should also be observed, based on the ideas of Winter and Kimmich (1982*a*, *b*). After Koenig et al. (1984). (*B*) An enlargement of the structured region of the data of Fig. 1 *A* after subtraction of the monotonic background. The dotted curves are Lorentzians centered at 2.15 and 2.82 MHz, each with the full linewidth at half height of 0.34 MHz. The solid curve is their sum.

DATA

Fig. 1 *A* shows the $1/T_1$ NMRD profiles of tissue water protons of an in vitro sample of rat heart (Koenig et al., 1984), before and after partial deuteration. The underlying monotonic variation of $1/T_1$ is clear, as is a region centered near 2.5 MHz that has unusual structure. The arrow at 0.67 MHz indicates where additional structure would be expected on the basis of the work of Winter and Kimmich (1982*a*, *b*). The structured region is shown enlarged in Fig. 1 *B*, in which the monotonic background, as represented by the smooth curve through the data of Fig. 1 *A*, has been subtracted. It is seen that the peaks can be resolved into the sum of two Lorentzians, peaked at 2.15 and 2.82 MHz, with full widths at half maxima of 0.34 MHz. (The resolution into Lorentzians both here and in Fig. 3 below was by trial and error, entirely adequate for the present purposes.)

There can be little question but that the peaks relate to transitions of the ^{14}N nuclei (with spin $I = 1$) of the heart muscle protein (Winter and Kimmich, 1982*a*, *b*), which have nuclear quadrupolar energy levels as shown in Fig. 2: two upper levels, linear combinations of states characterized by the magnetic quantum numbers $m = \pm 1$ and split because of anisotropy in the environment, are separated from the $m = 0$ ground state (Abragam, 1961). The two peaks in Fig. 1 *B* are associated with the transitions ν_1 and ν_2, Fig. 2. The peak corresponding to $\nu_3 = \nu_2 - \nu_1$ is seen clearly in the next figure.

Fig. 3 shows the ^{14}N peaks in the $1/T_1$ NMRD profile of solvent water protons of a homogenate of calf lens nucleus, the central part of the lens (Clark et al., 1981/1982). Other than the membrane fragments (which

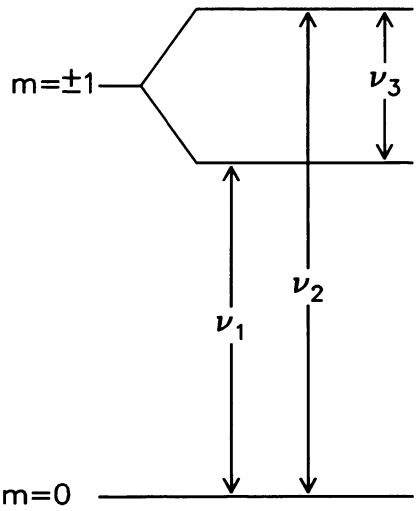

FIGURE 2 Energy levels of ^{14}N nuclei due to quadrupolar interactions with local electric field gradients. The two levels to the left occur for axial symmetry. The upper level splits when the axial symmetry is broken, as shown to the right. Additional splittings due to the magnetic dipole moments of the ^{14}N nuclei are small at the fields considered here, and have not been included. The lower level can be characterized by the magnetic quantum number $m = 0$; the upper states are orthogonal linear combinations of $m = \pm 1$, and the three possible transitions are indicated.

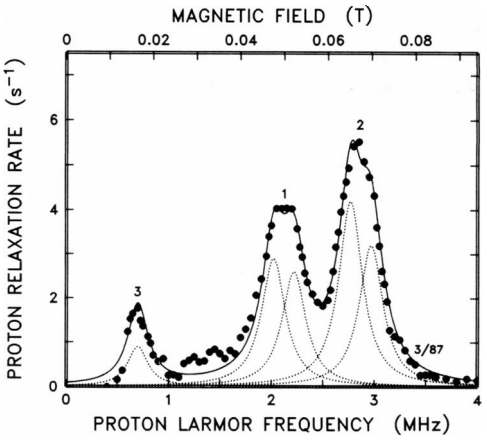

FIGURE 3 The enlarged $1/T_1$ NMRD profile of a homogenate of the nucleus of calf eye lens, (34% vol/vol, 25°C), essentially a concentrated solution of the lens proteins (crystallins), obtained as in Fig. 1 *B*. The dotted curves are two sets of Lorentzian triplets centered at 0.75, 2.02, 2.77, and 0.75, 2.22, and 2.97 MHz, with full linewidths of 0.24 MHz for the lower field (superimposed) peaks and 0.28 MHz for the higher field peaks. The triplets are labeled to correspond to the transitions indicated in Fig. 2. The solid curve is the sum of the Lorentzians. As expected from the model (cf., Fig. 2), the difference in frequency between the upper peaks of each triplet has been constrained to equal the lower peak frequency. Data after Beaulieu et al. (1987).

should not contribute appreciably to $1/T_1$), the sample is essentially a concentrated protein solution (with, coincidentally, a protein density corresponding quite closely to that of red blood cells). The quadrupolar peaks (not observed in concentrated hemoglobin solutions (Lindstrom et al., 1976)) are extremely large in the lens homogenate, almost 10-fold greater in absolute magnitude than those of rat heart muscle. In Fig. 3, the data are shown resolved into two sets of triplet transitions, ostensibly corresponding to two classes of NH sites with comparable concentrations, with the same anisotropy splitting $\nu_3 = 0.75$ MHz but with slightly different values for ν_1 and ν_2: 2.02 and 2.77 MHz for one, 2.22 and 2.97 MHz for the other. For both sets of transitions, ν_3 was set equal to $\nu_2 - \nu_1$ to be consistent with theory (see below). The full widths at half maxima are 0.24 MHz for the low field peak and 0.28 MHz for the high field peaks. There is additional structure in the region 1.1–1.6 MHz and at 3.2 MHz which, though small, appears real (Beaulieu et al., 1987), and is not addressed theoretically here.

THEORY

A Model for the Cross Relaxation Pathway

Each amino acid of the lens proteins (known as crystallins, and essentially 100% antiparallel β-sheet) contains an NH backbone moiety, most of which are hydrogen bonded to a carbonyl oxygen of the neighboring chain of the β-sheets of the crystallins (cf., Lindley et al., 1985). Taking a molecular mass of ~110 D for a typical amino acid, or six times that of water, a protein density of 0.73 and water proton molarity of 110, gives 7 M for the NH molarity, or ~0.06 that of water. We assume, because of the openness of the β-sheet structure, that essentially all the backbone NH groups are accessible to solvent. Now assume that (at least) one solvent molecule is also hydrogen bonded to each carbonyl oxygen (schematized in Fig. 4 for one NH moiety), which will then have one proton a distance from

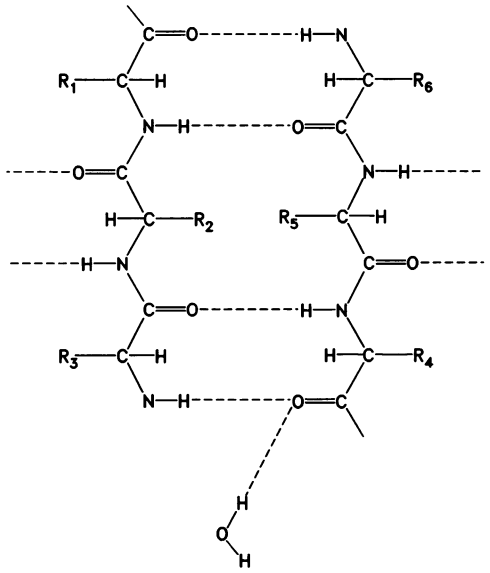

FIGURE 4 A schematized depiction of hydrogen bonding in an antiparallel β-sheet. The hydrogen bonding of an NH proton to a neighboring carbonyl oxygen is shown by a dashed line, as is the bond of a rapidly exchanging (~3×10^9 s^{-1}) water molecule bound near an NH moiety. The two hydrogen-bonded protons are sufficiently close to each other to interact magnetically as they would in a water molecule. The correlation time would be the lifetime of the bond which, though short, is much longer than the (rotationally determined) correlation time in water. Thus, the effective water proton–NH proton coupling would be enhanced. Resonant transfer of energy to the ^{14}N nuclei at level crossings accounts for the peaks in the NMRD profiles.

the NH proton comparable to the proton–proton separation in a molecule of water. Whenever the NH proton relaxes rapidly (which will be the case of level crossings), the proton–proton interaction between the two protons hydrogen bonded to the same oxygen (the NH proton and that of the water) will relax the proton of the exchangeable water. The rate will be 0.4 s^{-1} (that of water protons at 25°C) reduced by the factor 0.06, but also augmented by a factor due to the increased correlation time which, for the exchanging water, is the lifetime of its hydrogen bond. This can introduce a factor of two orders of magnitude or more (Bennett et al., 1987), increasing the correlation time from ~5×10^{-12} to ~3×10^{-10} s (see below), giving relaxation rates for the solvent protons in the range 1–10 s^{-1}, in excellent agreement with the peak rates in Fig. 3.

The relaxation rates of the NH protons can readily be estimated using the standard theory of relaxation of a pair of unlike spins, one (the ^{14}N) assumed to relax rapidly. Though usually applied to relaxation of protons by electronic moments, it works well here, giving rates sufficiently rapid so that the NH protons can be relaxation sinks for nearby water protons yet sufficiently slow so that the ^{14}N nuclei can always be considered at thermal equilibrium. For this case, because of the resonance condition associated with level crossing, the linewidths of the peaks, Figs. 1 *B* and 3, represent the transverse relaxation rates of the ^{14}N nuclear moments. These values appear quite reasonable

when compared with data for ^{14}N quadrupole relaxation in solutions of small ^{14}N-containing molecules (cf., Blinc et al., 1972), which were observed by a sensitive double resonance technique, but are broader than the ^{14}N lines that Kanazawa et al. (1979) could observe directly.

Theory for the NH Pair. The approach we take for estimating $1/T_1$ of an NH proton would be that developed earlier (Koenig, 1978), in which longitudinal relaxation is calculated by realizing that a precessing NH proton produces a radio frequency magnetic field at the position of its ^{14}N nucleus that, at the level crossings, causes a resonant absorption of power by the ^{14}N. The ratio of this power absorption to the steady state energy stored in the proton because of the radio frequency field imposed on it at the proton resonance (in turn, produced by the precessing magnetization of a neighboring water proton) gives $1/T_1$ immediately in terms of equations for the absorption spectra of the ^{14}N nuclei. Thus, for the peak corresponding to each ν_i, where $i = 1$, 2, and 3, we can obtain expressions for each of the several resonant contributions to $1/T_1$. The main difference from the earlier work is that the transitions in the present case, though occurring because of magnetic dipolar coupling between the ^{14}N and proton, involves ^{14}N quadrupolar levels and therefore these transitions require linearly rather than circularly polarized excitation. From Eq. 10 of Koenig (1978), $1/T_{1NH}$, the relaxation rate of the NH proton, is

$$\frac{1}{T_{1NH}} = 3.5C\tau_{c2}\left[\frac{1}{1 + (2\pi\nu_i - \omega_I)^2\tau_{c2}^2}\right] + 3.5C\tau_{c2}\left[\frac{1}{1 + (2\pi\nu_i + \omega_I)^2\tau_{c2}^2}\right] + 3C\tau_{c1}\left(\frac{1}{1 + \omega_I^2\tau_{c1}^2}\right), \quad (1a)$$

where

$$C = 4\gamma_N^2\gamma_I^2\hbar^2/15r^6. \quad (1b)$$

Here, $\omega_I = \gamma_I H_0$ is the proton angular Larmor frequency, H_0 is the applied static field, γ_N and γ_I are the ^{14}N and the proton gyromagnetic ratios (0.31 and 4.26 kHz per Oersted respectively), r the N-H distance (1.0 Å), and τ_{c1} and τ_{c2} are correlation times that, in the present case, are the longitudinal and transverse relaxation times of the ^{14}N nuclei. The first bracketed term of Eq. 1a is our present interest because of the resonant denominator; at $\omega_I = 2\pi\nu_i$, one obtains

$$1/T_{1NH}\,(\text{peak}) = 7.7 \times 10^8\tau_{c2} \simeq 770\ \text{s}^{-1}, \quad (2)$$

where we have used an approximate linewidth (Fig. 3) of 0.3 MHz, corresponding to $\tau_{c2} = 10^{-6}$ s. Assuming that τ_{c1} and τ_{c2} are comparable, the relaxation rate of an NH proton at a level crossing is never so rapid as to perturb the equilibria of the ^{14}N nuclei, yet is sufficiently rapid so that it can readily act as a relaxation sink for a nearby proton.

A few subtle points have been glossed over in the above derivation, which now need mention. First, the upper levels in Fig. 2 split in the presence of a magnetic field to an extent that depends on the orientation of the N-H bond with respect to the external static field. However, γ_N is relatively small, and when the effect of the splitting is averaged over all directions it contributes no more than a 5% broadening to the effective widths of the transitions ν_1 and ν_2; this correction has been ignored. Second, as pointed out by an anonymous reviewer, we have neglected the N-H magnetic dipole–dipole interaction, which introduces a term into the Hamiltonian with an energy of ~0.2 MHz. Third, not all of the transitions ν_i occur with the same intensity for a given orientation of the N-H bond with respect to the field. For example, ν_3 is a $\Delta m = 0$ transition whereas the other two require $\Delta m = \pm 1$. Reasoning from Fig. 1 of Koenig (1978), it can be shown, for example, that for the static field normal to the N-H bond, the ν_3 transition is forbidden. It is only by invoking the liquid properties of the samples, and averaging over all orientations of the solute protein, that Eq. 1a follows.

The NH . . . H Interaction. The large value of $1/T_{1NH}$ of an NH proton at level crossings justifies regarding it as a relaxation sink for nearby protons, in particular those on waters hydrogen bonded to the same carbonyl oxygen as is the NH proton, as schematized in Fig. 4. As noted above, these two bonded protons have approximately the same dipolar interaction as the two protons of water. However, the correlation time for the proton of the protein-bound water is the lifetime of the bond and that for water protons is one-third the rotational relation time of water, or $\sim 5 \times 10^{-12}$ s. There are few data on the lifetime of hydrogen bonds; the issue is rather complex and has been surveyed recently by Bennett et al. (1987). What is perhaps most germane is that, for homogeneous solutions of globular proteins, there is a relatively small contribution to the NMRD profile that does not disperse until relatively high fields, ~300 MHz, and which has been associated with rapid exchange of water molecules from the first hydration layer of the solute proteins (Koenig and Schillinger, 1969; Hallenga and Koenig, 1976). This high correlation frequency, which corresponds to a correlation time of 3×10^{-10} s, sets an upper limit to the lifetime of a "typical" hydrogen bond associated with exposed protein surfaces. If this limit can be taken as an estimate of the lifetime of the carbonyl-bound water, Fig. 4, then (as argued above) the magnitudes of the observed peaks in the lens homogenates can be explained quantitatively. This conclusion contains the important, though implicit, assumption that the solvent water has access to essentially all the backbone NH moieties, a circumstance that may be unique to crystallins (Lindley et al., 1985; Slingsby, 1985; Winstow et al., 1983), and associated with a specialized tertiary and quanternary structure (Delaye and Tardieu, 1983) that provides the high transparency of lens tissue.

DISCUSSION

The resolution of the data, Fig. 3, into a pair of triplets suggests that there are two major classes of NH groups, of comparable populations, that contribute to the ^{14}N peaks in the $1/T_1$ NMRD profiles of calf lens nuclear homogenates. It is premature to conjecture whether this is due to the known heterogeneity of the lens crystallins; to two classes of NH environments in any given crystallin form; or, as noted by the reviewer, to the N-H magnetic dipole–dipole interaction. In this connection, it should be noted that the class of NH moieties, Fig. 4, with their bonds within the structure are chemically inequivalent to those with their bonds oriented towards the edges of the figure unless there is mirror symmetry about the plane of the figure, because of the pleating of the β-sheets.

It is interesting to note that the linewidths of the underlying peaks for both the rat heart muscle and lens proteins are quite similar, and at least eight-fold wider than those observed by Blinc et al. (1972) by level crossing experiments for ^{14}N resonances in solutions of cytosine at 24°C. However, such significantly narrower lines are to be expected for the small solute cytosine than for the large proteins (in the motional narrowing limit); however, to quantitate this point is beyond the present concerns.

Several additional points are worth noting. One is that the nonresonant terms, Eq. 1a, are of little importance. The first of these will always be very small compared with the peaks—themselves small compared with the monotonic background—since $2\pi\nu_i\tau_{c2} \ll 1$. The last term will add a minor monotonic component to the NMRD profile at the lowest fields. The second point is the effect of deuteration. Both in rat heart, Fig. 1 *A*, and in the lens homogenates (Beaulieu et al., 1987), the entire NMRD profile decreases in magnitude in proportion to the decrease in proton fraction of the solution. The explanation is straightforward in terms of the model proposed here: a proton on a water that is hydrogen bonded to the carbonyl will have its $1/T_1$ reduced at all fields by a factor equal to the probability of interacting with an NH proton. Since deuterons compete equally with protons for the ^{14}N nites, this reduction will be linear in the solvent proton fraction, as observed. In addition, the present model would predict that deuteration could introduce other peaks into the NMRD profiles that arise from level crossings of the proton of the hydrogen bonded water with the quadrupole splitting of the deuteron of ND. Indeed, such a peak, at 0.15 MHz, has been recently reported (Kimmich et al., 1986), which gives addition support to the present model. The final point is that the small amount of structure in the relaxation spectra in the range 1–1.6 MHz and at 3.2 MHz near the foot of the major peak appears real. Moreover it can be measured with greater precision. Judging from the results of Blinc et al. (1972) for the NH_2 group of guanine, these features arise from interactions of the NH protons with another proton in a well defined geometry, yet to be identified. Of particular interest here is that, from the results of Beaulieu et al. (1987), these minor features were less apparent when the temperature was lowered below that at which a phase separation occurs and the sample becomes opaque, a model for cataract formation (Tanaka and Benedek, 1975). In fact, these minor features are the only aspects of the NMRD profile of lens homogenates reported so far that appear sensitive to the extent of long range ordering of the lens proteins.

It should be re-emphasized that the present work addresses the longitudinal relaxation rate of solvent water protons because these predominate in the samples of interest; in the lens (as noted above), they are 17-fold more numerous than the (exchangeable) NH protons. In earlier observations of ^{14}N peaks in proteins (summarized in Kimmich et al., 1986), the samples were either lyophilized or slightly hydrated. In the first case, NH protons are only observed in a solid-state environment. In the second, it is noted (Kimmich et al., 1986) that "the hydration water (~55% of the exchangeable hydrogen nuclei) directly or indirectly via spin exchange sense this type of (level crossing) interaction." That is, until the present work, there has been no quantitative theory of ^{14}N peaks applicable to liquid samples.

In summary, we have presented an explicit relaxation pathway to explain the ^{14}N peaks in the NMRD profiles of water protons of rat heart muscle and calf lens homogenates, and applied relaxation theory quantitatively to this model. The agreement of model and data is very gratifying, particularly since many subtle aspects of the present considerations all combine to create a self-consistent view of the cross relaxation phenomena that give rise to the peaks. On the basis of this agreement, one expects that refinement of such data—that for the lens homogenate being the first for soluble proteins—could lead to better understanding of the organization of crystallins in vitro and perhaps in vivo.

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