STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. II. NMR RELAXATION TIMES OF WATER PROTONS IN AQUEOUS SOLUTIONS OF GELATIN AND OXYGEN-CONTAINING POLYMERS WHICH REDUCE THE SOLVENCY OF WATER FOR NA^+, SUGARS, AND FREE AMINO ACIDS

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• *This communication reports our study of the NMR relaxation times, T_1 and T_2 of water protons in aqueous solutions of bovine serum albumin, gelatin, polyvinylpyrrolidone, poly(ethylene oxide), andpolyvinylmethylether over a wide concentration range. In contrast to solutions of gelatin and bovine serum albumin, the T_1/T_2 ratio of the threesyntheticpolymers are close to unity over the entire range studied. When combined with earlier-reported data of water made "non-solvent" to Na salts, the present data provided the basis for calculating the T_1 and T_2 as well as the rotational correlation time τ_c of the "non-solvent" water. It was shown that only a modest increase by a factor of about 3 of τ_c is enough to produce water that is "non-solvent" for Na citrate and sulfate. The new data reconciles NMR data of living cells with the theory of the cell water given in the association-induction hypothesis. The variability of τ_c of "non-solvent" water also offers explanations of apparently conflicting conclusions on the physical state of cell water from dielectric measurements.*

INTRODUCTION

The'development of the theories and techniques of NMR spectroscopy opened ways of studying living cells never known before. One subject of interest concerns the most abundant and ubiquitous component of all living cells: water. The central question is, "Does the bulk of water in living cells exist as free water as postulated in the membrane (pump) theory?" or "Does the bulk of cell water exist in the state of polarized multilayers and suffer rotational as well as diffusional motional restriction as suggested in a more recent theory of the living cell, the association-induction hypothesis (Ling, 1965a, 1965b, 1967, 1972, 1977, 1979a, 1979b, 1981; Ling and Sobel, 1975)?" In testing these alternative theories, a potentially useful approach involves the measurement of the spin-lattice relaxation time of the water protons, also called T_1, and the spin-spin relaxation time, also called T_2. The theory of NMR describes the magnitude of T_1 and T_2 as functions of the correlation time (τ_c) — which is the characteristic time of the rotation of a molecule or the time of its diffusion into its next position (Bloembergen et al, 1948; Kubo and Tomita, 1954; Solomon, 1952). When water molecules turn freely as in liquid water, τ_c of the water protons is short (2.6 X 10^{-12} sec.) (25° C) (Smith and Powles, 1966); in this case, T_1 and T_2 of the water protons are nearly equal (see below). When water molecules freeze into ice, the τ_c becomes much longer. T_2 then becomes much shorter than T_1.

Since Odeblad et al, (1956) made NMR measurements of water protons in living cells, much information on this subject has accumulated (for review, see Hazlewood, 1979). In general, it has been found that T_1 of water protons in living cells is considerably longer than T_2, often by a factor of about 10.

This difference between T_1 and T_2 by itself may be taken to indicate an "ice-like" or quasi-crystalline structure of cell water. However an examination of other characteristics of cell water tells a different story. Thus, the self-diffusion coefficient of water in living cells differs as a rule from that of normal liquid water by a factor not more than 2 (Ling et al, 1967; Abetsedarskaya et al, 1967; Mild et al, 1972; Finch et al, 1971; for review see Hazlewood, 1979); while in ice, the self-diffusion coefficient of water is one million times slower than that of normal liquid water (Eisenberg and Kauzmann, 1969). Therefore little doubt exists that cell water cannot be literally ice-like.

Taking into consideration these and other findings, some workers in this field have adopted the view that the bulk of cell water is simply normal liquid water. The reduced T_1 and T_2 observed are seen as the consequence of rapid exchange of this normal liquid water with one or more small fractions of rotationally bound or otherwise effectively relaxing water molecules with much shorter T_1 and T_2 (Zimmerman and Brittin, 1957; Cooke and Kuntz, 1974).

Now if the bulk of cell water is truly normal liquid water, many "pumps" must be postulated for living cells (see Ling, Miller and Ochsenfeld, 1973) in order to account for the asymmetrical distribution of sugars, Na^+ (vs. K^+), etc., between living cells and their environments. Yet it has been shown that the cell does not have enough energy to cope with even one of the postulated pumps (Ling, 1962, Chapt. 8; 1965a; 1980a). Serious and probably insurmountable as this objection is, it is but one of a lengthening list of evidence against the membrane-pump theory (Ling, 1977, 1983a, 1983b). Clearly some other explanations must be found in order to explain all the relevant data.

In the early 70's when the NMR investigation of cell water was most vigorously pursued, the polarized multilayer theory was not well known, nor was there a verifiable "test tube" model for this theoretically postulated state of water. Fortunately the condition has improved. First it was shown on theoretical grounds that the polarized multilayer theory does not imply a high degree of motional restriction of the water involved. Indeed, even large and complexed hydrated molecules and ions like Na' only need to be reduced in their motional freedom by a factor of 10 to account for the observed degree of Na^+ exclusion (Ling, 1979a). Simple model systems have also been found in which the bulk of the water does indeed exhibit properties predicted by the polarized multilayer theory, including the ability to exclude Na', sucrose, and free amino acids (Ling et al, 1980a, b). One recalls that it is the maintenance of low levels of these solutes in cell water that led first to the introduction of the membrane-pump theory and then the polarized multilayer theory of cell water (Ling, 1965b).

The choice of solvency reduction as the criterion for the recognition and detection of water in the state of polarized multilayers offers various advantages. Direct relevance to cell physiology is one. Another advantage lies in the quantitative information about the affected water that the solute distribution study provides. That is, the true equilibrium distribution coefficient of a probe molecule (or q-value) or the apparent equilibrium distribution coefficient (or p-value) (Ling and Sobel, 1975), can tell us quite unambiguously the minimal amount of the water that has been altered by the condition or substance that brings about the multilayer polarization. Studies of a family of polymers and proteins has shed light on the mechanism whereby these polymers and proteins change the properties of water (Ling et al, 1980a, b). Thus all the effective ones contain oxygen atoms at distances roughly equal to that of two water diameters apart. They are all "extended:" the oxygen atoms are not tied up

in one or another form of macromolecular H-bonds and thus are available to interact with the bulk phase water.

Among the polymers that affect water properties, gelatin has a long history, dating back to before Thomas Graham (1861) who used it to represent colloids. This unusual protein falls into the category of an NP-NP-NP system (Ling, 1972): its positively charged polypeptide NH (P) and negatively charged CO (N) groups are held in an extended and exposed condition due to the possession of many helix-breaking amino acid residues: glycine, proline, and hydroxyproline (Veis, 1964; Chou and Fasman, 1974). Other simpler water-polarizing polymers include polyvinylmethylether (PVME), polyvinylpyrrolidone (PVP), and poly(ethylene oxide) (PEO); all belong to what is called an NO-NO-NO system as these polymers possess no P sites but only N sites in the form of oxygen atoms with two negatively charged lone pairs of electrons. The sites between each pair of N sites are vacant and are referred to as 0 sites.

Following the recognition of the water-property-altering power of these polymers, we (and others) conducted investigations of several other physico-chemical attributes of water in this unusual physical state. Besides its solvency-reducing effects already mentioned (Ling et al, 1980a, b; Ling and Ochsenfeld, 1983), other studies include the osmotic activity (Ling, 1983a, c); swelling and shrinkage (Ling 1980b; 1983d); freezing and thawing (Ling and Zhang, 1983); and quasi-elastic neutron scattering (Rorschach, 1983). This communication adds the results of our measurements of the NMR relaxation times T_1 and T_2 of similar polymer- and gelatin-dominated water and of water in solutions of a protein that does not have the solvency-reducing power, native bovine serum albumin (BSA). A brief note was published earlier (Ling and Murphy, 1982).

MATERIALS AND METHODS

Materials. Polyvinylmethylether (PVME) (Gantrez M-154®), Batch 185, was from GAF Corp., NY: poly(ethylene oxide) (PEO), (Polyox-205), from Union Carbide, NY: polyvinylpyrrolidone, PVP-360, from Sigma Chemical Co., St. Louis, Lot 57C-0071; and gelatin (Type I, approx. 300 Bloom), (swine skin) also from Sigma.

NMR relaxation time measurements. Water proton NMR relaxation times T_1 and T_2 were measured with a coherent CPS-2 NMR pulse spectrometer (Spin Lock, Ltd., Port Credit, Canada) operating at a resonance frequency of 17.1 MHz. T_1 was determined with a $180°$-τ-$90°$ pulse sequence; care was taken to insure reestablishment of equilibrium between readings by allowing at least five times T_1 in between readings (see Farrar and Becker, 1971). T_2 was determined with Carr-Purcell spin echo methods. The sample temperature was $25° \pm 1.0°C$.

Preparation of paramagnetic ion-free polymers. All glassware — including drying vessels and transfer pipets — which came into contact with polymers, were acid washed (concentrated HCl or aqua regia) and triple rinsed in distilled water. A 5% polymer solution was prepared by floating dry polymer over water in a wide vessel and allowing it to shake gently until dissolved. Washed Chelex 100 resin (Bio-Rad, Richmond, Calif., 100-200 mesh, sodium form) was added to the solution and allowed to shake with the polymer solution until the solutions were free of paramagnetic ions (2-8 days depending on polymer). To assay for paramagnetic impurities the solutions were dried and the dry residues ashed at 600°C in a muffle furnace. The T_1 of a dilute acid extract of the ashes when compared with that of the acid itself determines the presence or

absence of the paramagnetic impurities. No attempt was made to remove oxygen in the samples.

RESULTS

Bovine serum albumin and gelatin. Figure 1 gives illustrative examples of plots of $\ln(A_\infty - A_\tau)$ against varying values of τ for solutions of purified bovine serum albumin (BSA), gelatin as well as the polymers **PEO, PVP,** and **PVME.** A, is the initial amplitude of the free induction decay signal following the 90° pulse at time τ. A_∞ equals A_τ when $\tau \to \infty$. As a rule, the data can be adequately described by a single slope, equal to $-(T_1)^{-1}$. Figure 2 illustrates the Carr-Purcell plots for the determination of T_2. The logarithm of the echo height at time t, $\ln A_t$, is linearly related to t also with a single slope, equal to $-(T_2)^{-1}$. With these methods the data on T_1 and T_2 were obtained for solutions of BSA and gelatin at different concentrations and shown in Figures 3 and 4 respectively. The T_1's are considerably longer than T_2 throughout the entire range of concentrations studied. The differences between T_1 and T_2 are most pronounced at the lower H_2O contents where

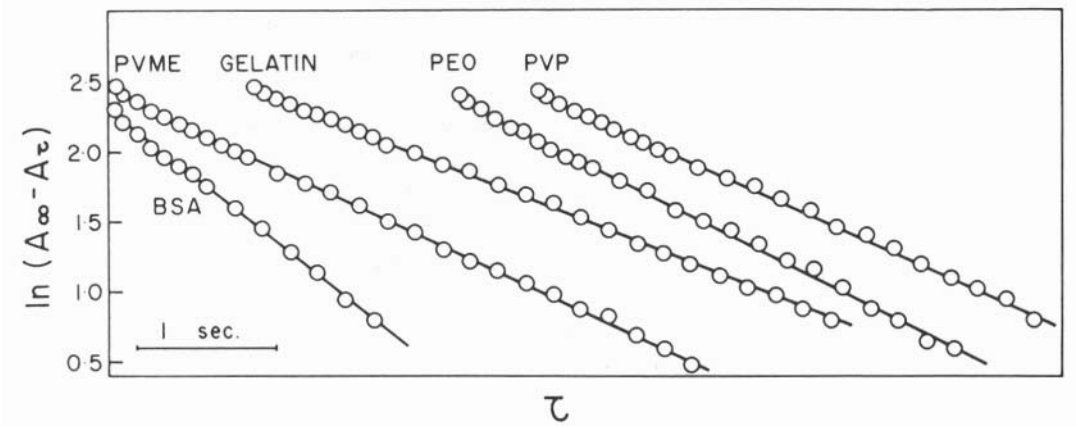

FIGURE 1. Plots of ln ($A_\infty - A_\tau$) against τ. A_∞ is the initial amplitude of the free induction decay signal following the 90° pulse at time τ. A_τ is A_τ as $\tau \to \infty$. The data illustrate that the plots can be filled with a single slope, equal to $-(T_1)^{-1}$. The concentrations were: BSA (10.0%); gelatin (2.94%); PVP (5.66%); PEO (7.67%); PVME (8.6%).

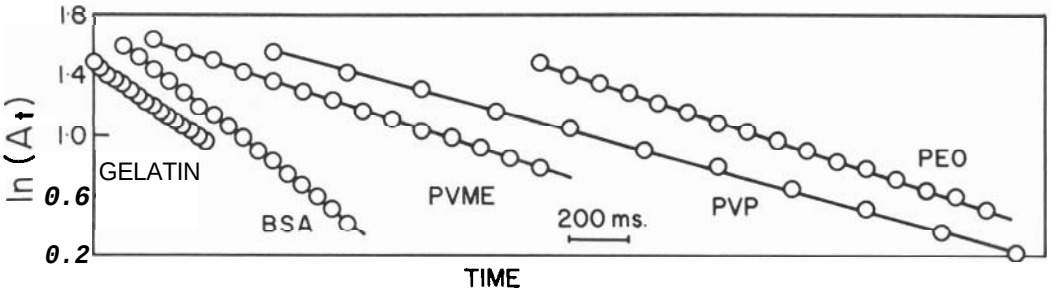

FIGURE 2. Carr-Purcell plots of ln A_t against t where A_t is the echo height at time t. The data illustrate that the plots can be fitted with a single slope equal to $-(T_2)^{-1}$. The concentrations were: BSA (8.3%); gelatin (8.0%); PVP (9.3%); PEO (6.1%); PVME (9.9%).

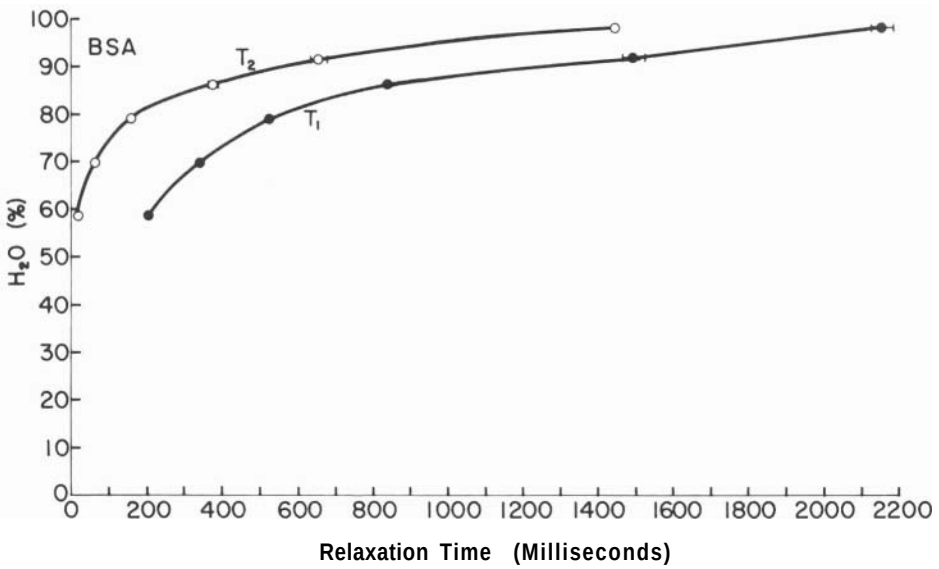

FIGURE 3. T_1 and T_2 of water proton in aqueous solutions of bovine serum albumin plotted against their water contents.

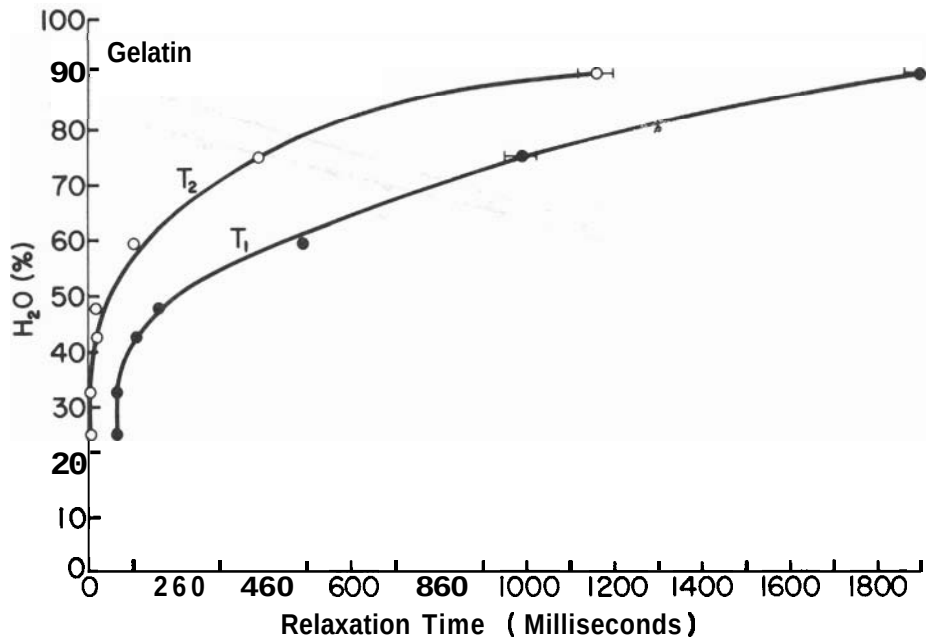

FIGURE 4. T_1 and T_2 of water proton in aqueous gelatin gel plotted against their water contents.

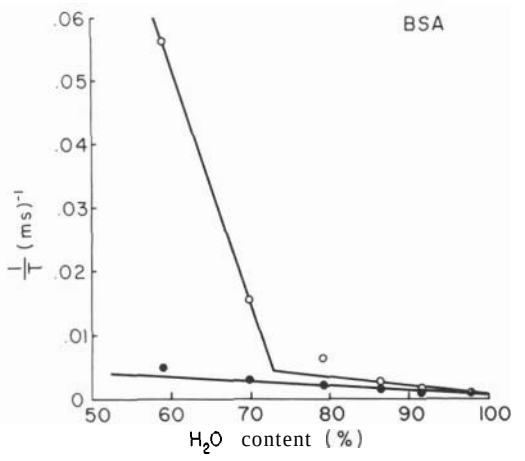

FIGURE 5. Plots of $(T_1)^{-1}$ (•) and $(T_2)^{-1}$ (o) against the water contents of different concentrations of bovine serum albumin (BSA). Data same as in Figure 3.

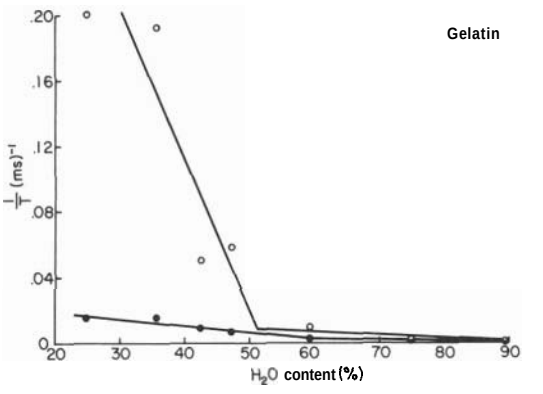

FIGURE 6. Plots of $(T_1)^{-1}$ (*) and $(T_2)^{-1}$ (o) against the water contents of different concentrations of gelatin gel. Data same as in Figure 4.

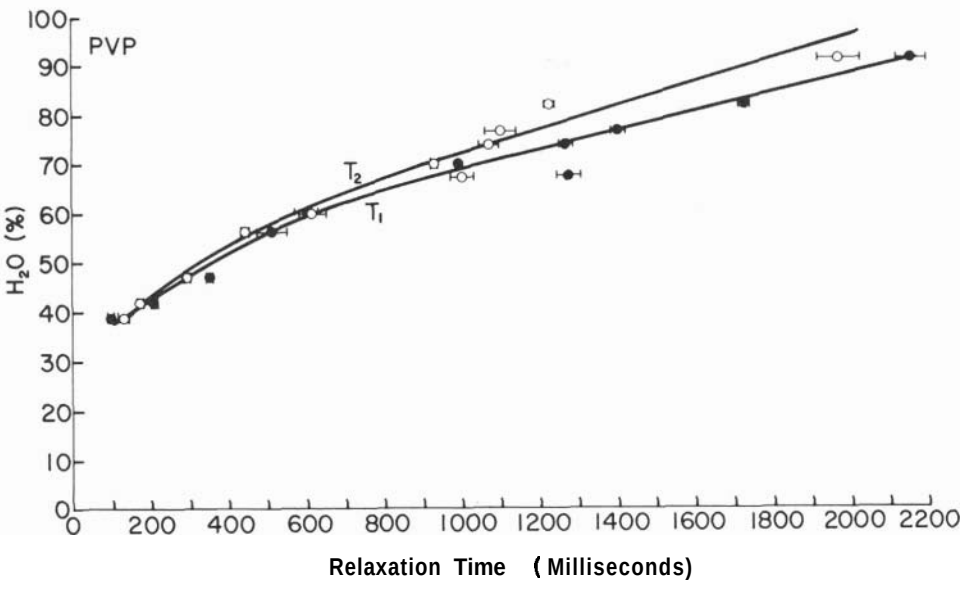

FIGURE 7. T_1 and T_2 water protons in aqueous solutions of polyvinylpyrrolidone (PVP) against their water contents.

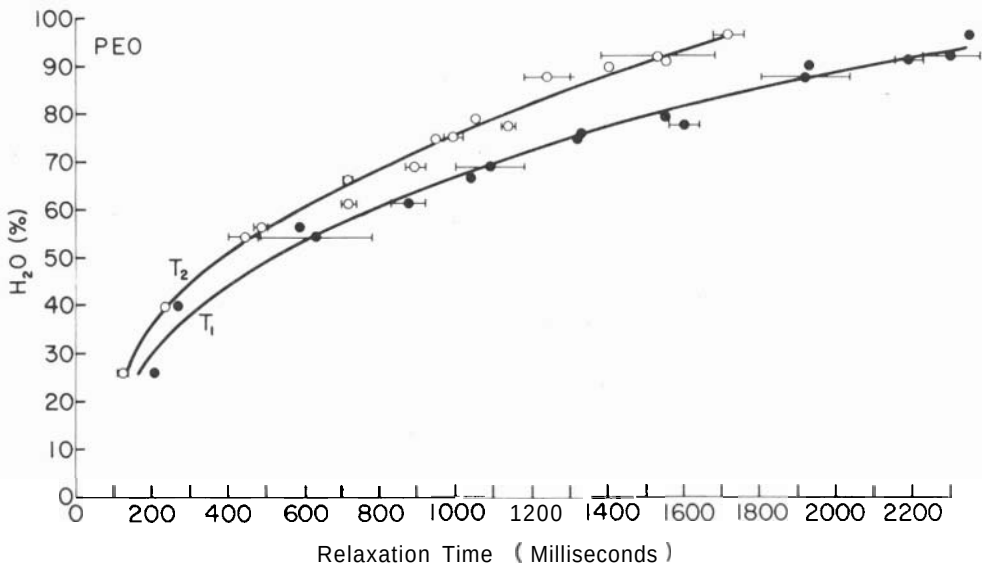

FIGURE 8. T_1 and T_2 of water proton in aqueous solutions of poly(ethylene oxide) (PEO) against their water contents.

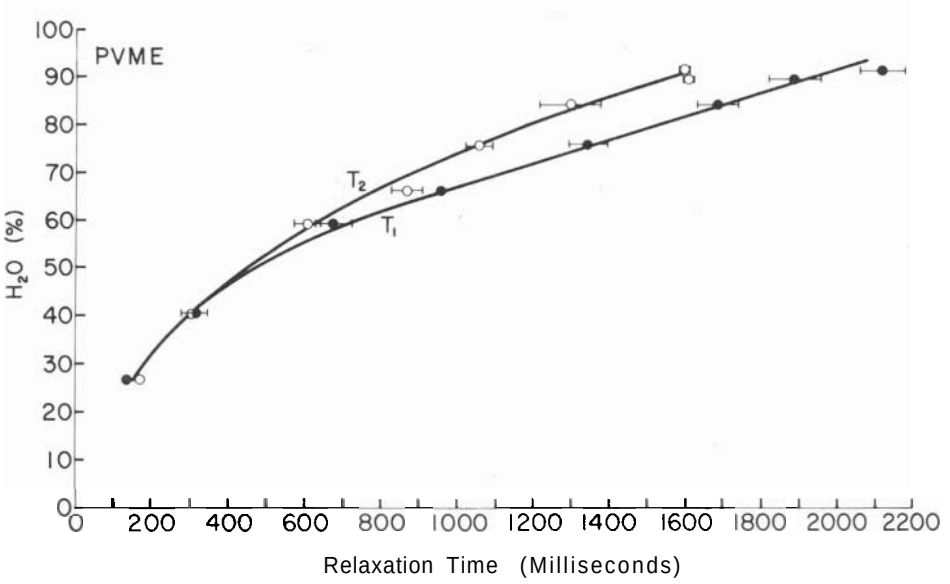

FIGURE 9. T_1 and T_2 of water protons in aqueous solutions of polyvinylmethylether (PVME) against their water contents.

both the T_1 and T_2 curves bend sharply. In Figures 5 and 6, the same data were plotted as $1/T_1$ and $1/T_2$ against the H_2O contents. Note that in each case the $1/T_1$ and $1/T_2$ abruptly diverge after the water content falls below a certain value: 72% for BSA and 51% for gelatin. In general these data are close to those reported earlier for isolated proteins (Cooke and Wien, 1971). However, quantitatively the curves for gelatin and BSA differ considerably. At the same concentrations, T_1 and T_2 of gelatin are much longer than those of BSA.

PVP, PEO ***and*** PVME. Figures 7, 8, and 9 show the T_1 and T_2 values from solutions of PVP, PEO, and PVME, respectively. The results differ markedly from those of protein solutions. First, the T_1 and T_2 curves are much closer together. The T_1/T_2 ratios of solutions of these polymers as given in Table 2 are not that far different from pure water at several pH's in air and in nitrogen as given in Table 1 (see also Meiboon et al, 1957; Meiboon, 1961). Figures 10, 11, and 12 show nearly equal rates of spin lattice relaxation and spin-spin relaxation represented by $1/T_1$

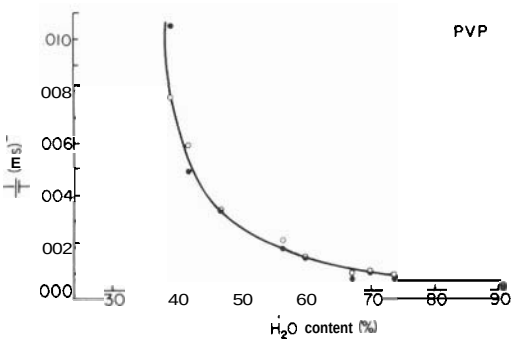

FIGURE 10. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of thesolutions of polyvinylpyrrolidone of different concentrations. Data same as Figure 7.

TABLE I. T_1 and T_2 of water protons of distilled water at different pH's in air or nitrogen.

Distilled Water	pH	T_1 (msec)	T_2 (msec)	$\frac{T_1}{T_2}$
Air	6.6	2550	1740	1.47
N_2	6.6	3200	2100	1.52
Air	3.5	2550	2150	1.21
N_2	3.5	3250	2700	1.20
Air	10.6	2600	2200	1.30
N_2	10.6	3325	2650	1.28

2567 ± 13.9

TABLE 2. The ratios of T_1/T_2 for BSA, gelatin, PVP, PEO, and PVME at various water contents. To save space, every 4th set of data from PVP and every second set of data from PEO were deleted. These deleted data are in essence similar to those retained.

BSA		Gelatin		PVP		PEO		PVME	
% Hz0	T_1/T_2	% H_2O	T_1/T_2	% H_2O	T_1/T_2	% Hz0	T_1/T_2	% Hz0	T_1/T_2
98.0	1.49	89.5	1.63	90.7	1.10	95.9	1.37	91.2	1.32
91.7	2.27	74.9	2.54	81.6	1.41	90.8	1.41	83.0	1.30
86.5	2.25	59.3	4.67	76.4	1.27	87.4	1.54	75.4	1.27
79.2	3.30	47.2	9.29	69.9	1.06	77.1	1.40	65.9	1.10
70.0	5.27	42.4	5.50	67.1	1.28	77.8	1.39	59.4	1.11
59.0	11.50	35.6	12.50	59.9	0.98	66.4	1.45	40.4	1.01
		24.8	13.00	41.8	1.20	56.3	1.20	26.8	1.82
				39.0	0.74	39.9	1.20		

and $1/T_2$ respectively, in marked contrast to the widely divergent $1/T_1$ and $1/T_2$ of the two proteins shown in Fig. 3 and 4, and Table 2.

DISCUSSION

Earlier Interpretations of NMR Data of Protein Solutions and of Living Cells. The theory of Bloembergen, Purcell and Pound (1948), Kubo and Tomita (1954) and Solomon (1955) permitted the calculation of the relaxation rates ($1/T_1$ and $1/T_2$) as a function of τ_c at a specific field frequency. For water protons in a magnetic field of 17.1 MHz (the frequency of our instrument), the relation between τ_c, T_1 and T_2 is illustrated in Figure 13. To be noted is that T_1 goes through a minimum, the value of which varies with the field frequency. At τ_c lower than that corresponding to the T_1 minimum, T_1 and T_2 are essentially equal; above it, they diverge. As mentioned earlier, the longer T_1 than the T_2 often observed for water protons in living cells and in solutions of a variety of isolated proteins have been interpreted as due to rapid exchange between a minor fraction of water protons with a very high value of τ_c and a much larger fraction with a much lower τ_c. Thus if the rate of this exchange is much faster than the NMR relaxation rates, then the observed NMR relaxation time T_{obs} would be a single value equal to the weighted average of relaxation times in the n different populations of water protons (Zimmerman and Britten, 1957):

$$T_{obs}^{-1} = \sum_{i=1}^{n} P_i \, T_i^{-1} , \qquad (1)$$

where P_i is the probability of the water proton being found in that specific ith population with T equal to Ti. Pi is usually taken to be equal to the size of the proportion of water belonging to that population.

Cooke and Kuntz (1974) explained the observed T_1 and T_2 of water protons in isolated protein solutions at 1 to 100 MHz on the assumption that the bulk of water is normal liquid water (Type I) with a τ_c equal

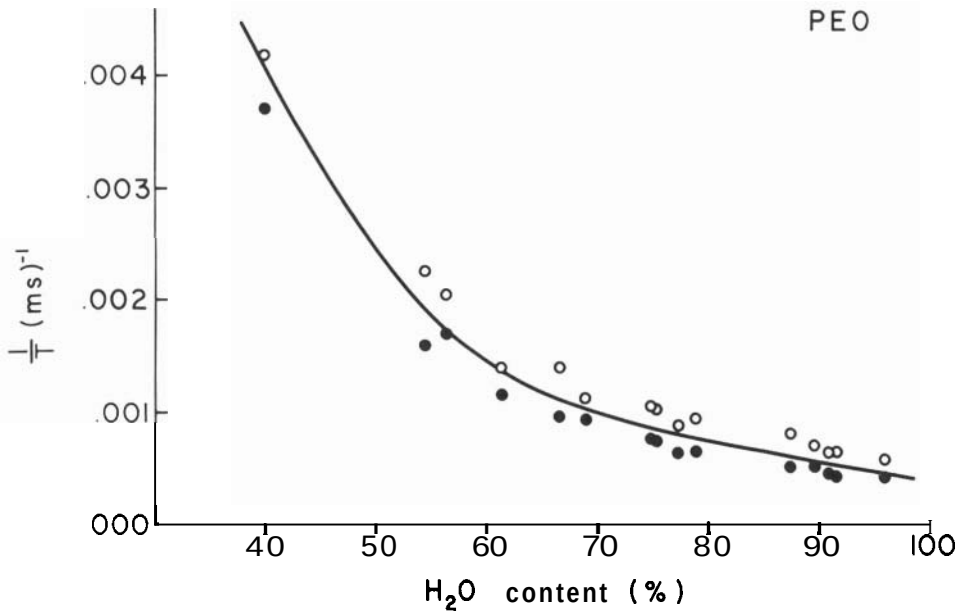

FIGURE 11. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of poly(ethylene oxide). Data same as in Figure 8.

to 3 X 10^{-12} sec. in rapid exchange with one fraction of hydrated water (Type II) of an amount equal to 0.3 to 0.6 g. H_2O/g. protein with a τ_c of 10^{-9} sec., and with another minute fraction (Type III) as little as 0.003 g. H_2O/g. protein with a τ_c of 10^{-6} sec. They then extended this conclusion to living cells.

Cooke and Kuntz's model of protein solutions and living cells in fact maintains that the observed T_1 is determined primarily by the Type II water and the observed T_2 by the Type III water. This masking of the T_1 and T_2 of the large fraction of Type I water by the relaxations of the minor fractions of water makes it difficult to learn much about the nature of the bulk phase water in these cases from the T_1 and T_2 data. Nevertheless, Cooke and Kuntz did draw the conclusion from their NMR studies that the bulk of water in living cells is normal liquid water. This conclusion was perhaps founded partly on the similarity in the behavior of protein solutions and living cells they studied, and partly on the assumption that if the bulk phase cell water is in the state of polarized multilayers, its τ_c would be much longer than that of normal water and the T_1 and T_2 would no longer agree with the values observed. The data on the polymer – dominated water presented here leads us to conclude that this assumption is not correct.

Estimates of τ_c of Polymer-dominated Water. PVP, PEO, and PVME are all long linear chains of similar bifunctional monomeric units joined end to end. There are no polar side chains, hence no Type II water. Nor is there the basis for postulating the existence of special sites that can powerfully restrict the rotational motional freedom of water molecules as suggested for Type III water in protein solution (Cooke and Kuntz). In agreement with these simple facts, T_1 and T_2 of water in solutions of these three polymers are essentially equal over the whole range of polymer concentration studied, much as they are in distilled water. A comparison of the data with Figure 13 shows

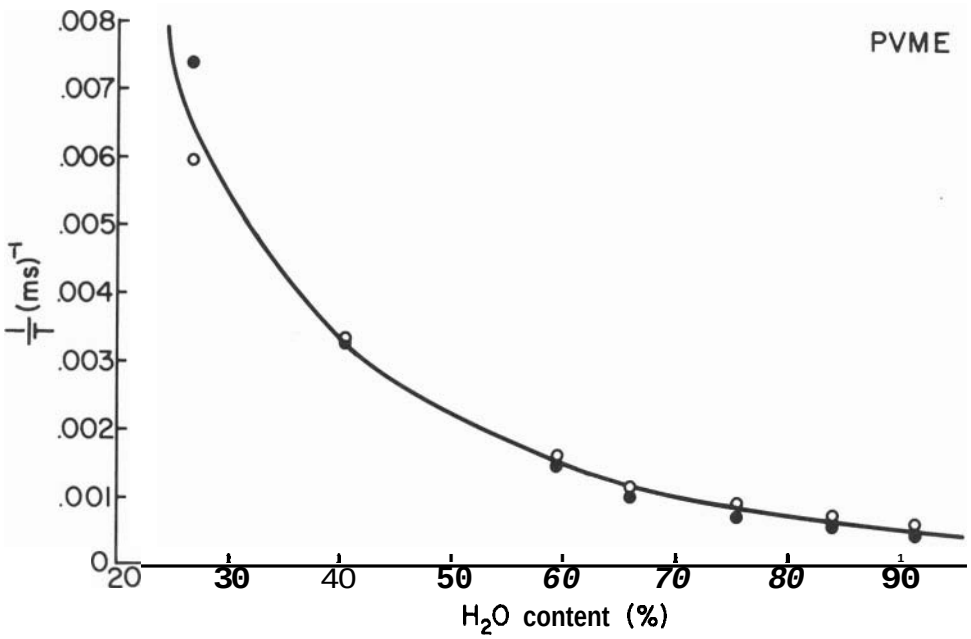

FIGURE 12. **Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of various polyvinylmethylether solutions. Data same as in Figure 9.**

that no significant population of water protons could have τ_c much longer than that corresponding to the T_1 minimum at 3×10^{-8} sec. Further analysis, to be described later, shows that the τ_c is in fact much shorter than this.

Since sites that theoretically could produce Cooke and Kuntz's Type II and III water cannot exist in these polymers, the reduced T_1 and T_2 when compared to those of liquid water can be explained in only two ways: All water at all polymer concentrations is influenced by the polymers; this is not impossible but rather unlikely. A more likely interpretation is that some water molecules are significantly affected by the polymers and the rest of the water molecules are normal liquid water.

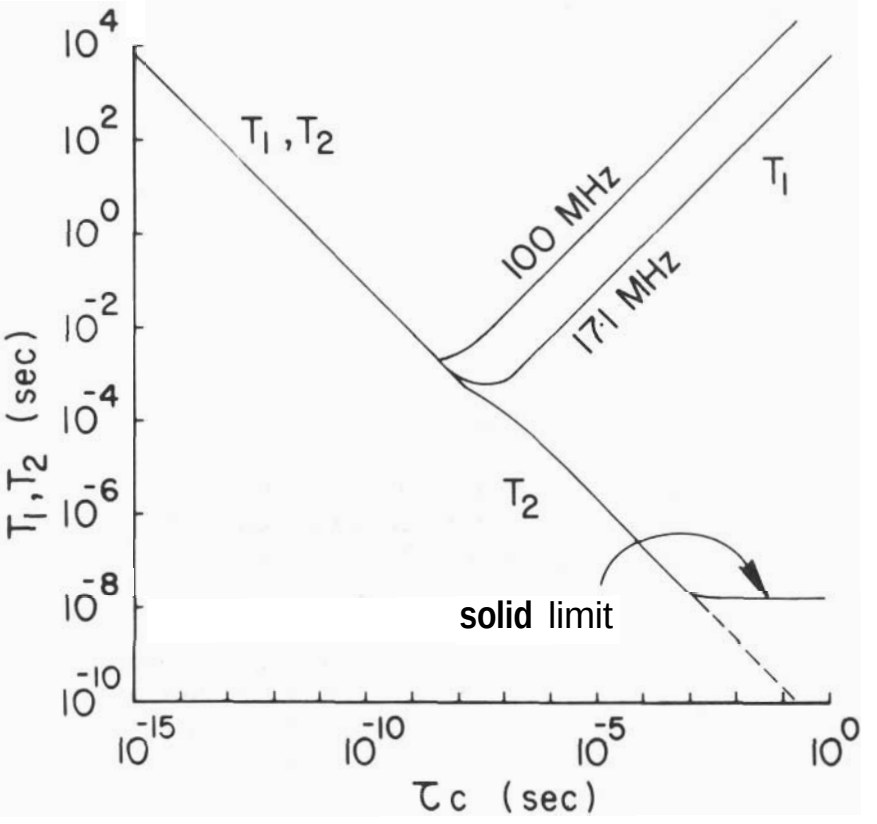

FIGURE 13. Theoretical curves of T_1 and T_2 of water proton at various rotational correlation times, τ_c, calculated from the equations of Bloembergen, Purcell and Pound (1948) as modified by Kubo and Tomita (1959):

$$(T_{1(\text{intra})})^{-1} = \frac{3\gamma^4\hbar^2}{10b^6}\left[\frac{\tau_c}{1+\omega_o^2\tau_o^2} + \frac{4\tau_c}{1+4\omega_o^2\tau_c^2}\right] \text{ and}$$

$$(T_{2(\text{intra})})^{-1} = \frac{3\gamma^4\hbar^2}{20b^6}\left[3\tau_c + \frac{5\tau_c}{1+\omega_o^2\tau_c^2} + \frac{2\tau_c}{1+4\omega_o^2\tau_c^2}\right]$$

where y is the magnetogyric ratio (equal to 5.585 for protons, h is the Plank constant $(h/2\pi)$; b is the interproton distance, ω_o is the Larmour frequency (17.1×10^6 Hz for our instrument). The value of $A = \frac{3\gamma^4\hbar^2}{20b^6}$ used was $0.83 \times 10^{10}\text{sec}^{-2}$.

Even though from the viewpoint of the polarized multilayer theory, there cannot be sharp boundaries between water molecules affected by the polymers and those that are not, it is, nevertheless, convenient to assume arbitrarily that water in the PVP, PEO, and PVME solution can be divided into two kinds. They are designated as polymer-altered water (PAW) and normal liquid water. Our next questions are: (1) "What part of the polymer molecules could have affected the water?" (2) "How much water is altered by the polymer at each polymer concentration?" and finally (3) "What is the correlation time, τ_c, of this polymer-altered water?"

1. ***What part of the polymer molecules alter the water?*** The structures of PEO and PVME are very simple; there are only two types of atoms or atomic groups: CH_2 and CH_3 on the one hand, oxygen on the other. There is no significant interaction between saturated hydrocarbons and water; indeed it is this lack of hydrocarbon-water interaction that creates the so-called "hydrophobic bonds." This leaves oxygen atoms as the primary seats of water-polymer interaction. There is other evidence supporting this conclusion. As an example, in his studies of amide-water interaction, Wolfenden (1978) found that the peptide oxygen, not the NH group, is the major site of interaction with water.

2. ***How much water is altered by the polymers at each polymer concentration?*** Due to the masking effect of any rapid exchange of magnetic energy among water molecules and between water and non-water molecules or atoms by spin diffusion, it is difficult to tell from NMR data how many water molecules in a complex system like a polymer solution have altered NMR relaxation times. However, it is here that the property of solvency reduction of the water is most useful. Thus if a p-value of 0.5 for a certain probe molecule is demonstrated, at least 50% of the water must be affected. In Tables 3 and 4, we have included the p-values for Na citrate of solutions of the two polymers PEO and PVME from Ling and Ochsenfeld (1983). Also cited in these tables are the estimated minimal amount of polymer-altered water given in percentage. Thus at 50% concentration, at least 90% of the water of both PEO and PVME solutions has lost its solvency for Na citrate. In 7.5% PEO solutions, only 13% of the water has been so affected while in 7.5% PVME 50% of the water has lost its solvency for Na citrate. Moreover, the altered water is not likely to have lost completely its solvency. In that case, the percentage of polymer-altered water will be even greater.

3. ***What is the rotational correlation time τ_c of the polymer-altered water?*** Once we know how much of the water in a particular concentration of PEO or PVME is made non-solvent to Na citrate, we can calculate the T_1 of this altered water from the measured T_1 of that polymer solution by making use of Equation 1. The fact that only a single T_1 (and T_2) was observed, justified the use of the rapid exchange model on which Equation 1 was based. For an illustrative discussion we may choose a 50% PEO solution. Here the measured T_1 taken from the data of Figure 8, and tabulated in Table 3 is 520 msec or 0.52 seconds. Since the un-degassed water measured with our spectrometer has a T_1 of 2.6 sec. (Table 1), the average T_1 of the polymer-altered water, represented as T_1^{PAW} is described by

$$\frac{0.92}{T_1^{PAW}} + \frac{0.08}{2.6} = \frac{1}{0.52}, \qquad (2)$$

where 0.92 and 0.08 are respectively the volume percentage of polymer-altered water and normal liquid water respectively. From this equation, a T_1^{PAW} of 487 msec. is calculated. This and other data are recorded in Table 3.

A survey of the T_1^{PAW} in Tables 3 and 4, reveals that, as a rule, its value increases as the percentage of polymer decreases. Based on the theoretical curve of Figure 13, we also estimated the rotational correlation time, τ_c, for the polymer-altered water. The τ_c values of T_1^{PAW} given in Tables 3 and 4 range from 7.6 X 10^{-12} second for 7.5% PVME to 4.88 X 10^{-11} for 50% PVME. Comparing these with the τ_c of normal liquid water (3 X 10^{-12} second) one finds that (i) it is longer but only by a very modest factor and (ii) the τ_c for the polymer-altered water ranges from 3 to 19 times slower than the τ_c of normal liquid water. We caution that the precise values of τ_c estimated must not be overly emphasized. First, the exact values of constants used in computing the curves are subject to some variation. Second, the theoretical curve of rotational correlation time we used refers only to the intramolecular dipolar interaction ($T_{1(intra)}$) while the observed longitudinal relaxation (T_1) originates from both $T_{1(intra)}$ and intermolecular interaction ($T_{1(inter)}$), involving largely diffusional motion. Estimates of from 25% (Abragam, 1961, p. 302) to as high as 50% (Emsley, Feeney, and Sutcliffe, 1965; Krynicki, 1966) of the T_1 of water proton may originate from the intramolecular effect. Since

$$T_1^{-1} = T_{1(intra)}^{-1} + T_{1(inter)}^{-1}, \qquad (3)$$

by ignoring the intermolecular contribution, we in fact somewhat overestimate the value τ_c. But this error is not very significant in the degree of quantitative accuracy we strive for now.

A New Interpretation of the NMR Data from Living Cells. Up to now, major disagreements exist on the subject of the physical state of water in living cells: From NMR studies some investigators claim the bulk of cell water is normal. Others objected to this view because of the insufficiency of energy to operate the membrane-pumps which the normalcy of cell water inevitably demands (for full discussion on this subject, see Ling, 1962, 1983b).

As mentioned earlier, part of the free-cell-water conclusion might have originated from an erroneous guess of what τ_c should be for water protons in the state of polarized multilayers. The experimental demonstration presented here shows that the τ_c of water

TABLE 3. The T_1^{PAW} and τ_c^{PAW} of estimated polymer – altered water (PAW) in PEO-water system. The amounts of PAW were obtained from the p-values for Na citrate and were taken from Ling and Ochsenfeld (1983). Method of calculations described in text.

PEO							
Polymer Content (%)	7.5	10	15	20	30	40	50
H_2O Content (%)	92.5	90	85	80	70	60	50
T_1(msec)	2280	2110	1800	1540	1110	780	520
$\rho_{Na\ citrate}$	0.87	0.73	0.51	0.39	0.22	0.13	0.08
Minimal polymer altered water (%)	13	27	49	61	78	87	92
T_1^{PAW}(msec)	1250	1400	1360	1220	947	707	487
τ_c^{PAW}(sec)	1.02×10^{-11}	9.2×10^{-12}	9.4×10^{-12}	1.06×10^{-11}	1.36×10^{-11}	1.81×10^{-11}	2.67×10^{-11}

fully able to exclude Na citrate may, nevertheless, be different from that of normal water by a modest factor of 3. This modest lengthening of τ_c opens the door toward a new interpretation of the NMR data of water in living cells.

First we must understand that the foundation for the conclusion of normal liquid cell water is of limited precision and involves assumptions that seemed reasonable but not proven. Thus Cooke and Kuntz estimated Type II water to range from 0.3 g. protein to twice that much. They also considered that Type II water is the non-freezable water. Kuntz et al (1969) showed that the non-freezable water in a BSA solution amounts to 0.4 g. H_2O/g. protein. It has long been established that 60% gelatin does not freeze even in liquid nitrogen (Moran, 1926). Thus the non-freezable water of gelatin is $40/60 = 0.67$ g. H_2O/g. protein. Comparing the two, one concludes that a gelatin solution has much more Type II water than one of BSA of equal concentration. With more Type II water, the T_1 of a gelatin solution should be considerably shorter than that of an equal concentration of BSA.

Now Cooke and Kuntz estimated the "intrinsic" T_1 for Type II water at 40 msec, a value corresponding to a τ_c of 10^{-9} sec. At this τ_c value, T_1 is independent of the field frequency (see Figure 13). Thus their data of 40 msec or .04 sec. estimated at 100 MHz can be directly applied to our measurements at 17.1 MHz. Using Equation 1 we have for a 20% solution of BSA and a value of 2.6 second for T_1 of normal liquid water, a predicted T_1 for water protons given by:

$$\frac{1}{T_1} = \frac{1-(0.2 \times 0.4)}{2.6} + \frac{0.2 \times 0.4}{0.04}, \quad (4)$$

$$T_1 = 426 \text{ msec.} \quad (5)$$

For a 20% gelatin solution,

$$\frac{1}{T_1} = \frac{1-(0.2 \times 0.66)}{2.6} + \frac{0.2 \times 0.66}{0.04}, \quad (6)$$

$$T_1 = 275 \text{ msec.} \quad (7)$$

Now let us compare these theoretical values with those actually measured. An examination of Figure 3 shows that T_1 of 20% BSA is 620 msec, which is not a good fit of the predicted value of 426 msec but perhaps acceptable. A much worse prediction is that of the gelatin solution. Thus T_1 of a 20% gelatin solution actually measured is not shorter than that of BSA as predicted. Rather it is longer considerably, at 1300 msec!

This rather severe discrepancy between theoretical predictions and experimental observations illustrates first that the non-freezable water may not necessarily be the source of T_1 reduction in all protein solutions. Second, we suspect that non-freezable

TABLE 4. The T_1^{PAW} and τ_c^{PAW} of estimated polymer altered water (PAW) in PVME-water system. The amounts of calculations described in the text.

PVME				
Polymer content (%)	5	7.5	10	15
H_2O content (%)	95	92.5	90	85
T_1 (msec)	2100	2050	1950	1750
$\rho_{Na\ Citrate}$	0.89	0.50	0.45	0.39
Minimal polymer-altered water (%)	11	50	55	61
T_1^{PAW} (msec)	1821	1690	1620	1450
τ_c^{PAW} (sec)	1.55×10^{-11}	7.6×10^{-12}	7.9×10^{-12}	8.9×10^{-12}

water may not be always equated with hydration water of polar side chains (Bull and Breese, 1968; Kuntz et al, 1969). Were it otherwise, there should be no non-freezing water in solutions of PEO, PVME, and PVP at all. None of these polymers contains polar side chains but they all show large amounts of non-freezing water as will be described in detail in a forth-coming paper (Ling and Zhang 1983).

From the data of Ling and Ochsenfeld (1983), one finds that the p-value for Na citrate in a 20% gelatin solution is 0.87. If one assumes that there are only two kinds of water in this gelatin solution, "non-solvent" water and normal liquid water, they would correspond, respectively, to 13% and 87% of the total water. Again using Equation 1, and the measured T_1 of 1300 msec for a 20% gelatin solution one finds that

$$\frac{1}{1.3} = \frac{0.87}{2.6} + \frac{0.13}{T_1^{PAW}}, \quad (8)$$

and

$$T_1^{PAW} = 0.300 \text{ second}. \quad (9)$$

A comparison with the T_1 of non-solvent water in the PEO and PVME systems at similar concentrations given in Tables 3 and 4 suggests that 300 msec may be too low a figure to be consistent with the PEO and PVME data. That is, in promoting water proton relaxation, gelatin may have in addition to what it shares with PEO and PVME, something extra. What can this extra element be? Probably polar side chains.

So we have come a full cycle back to the polar side chains as a major source of water proton NMR relaxation in proteins. Indeed, another compelling reason for its involvement is the even shorter T_1 for native BSA solution, which Ling et al (1980a, b) have shown to contain little "non-solvent water" for Na sulfate. However the role of polar side chains in causing water proton relaxation may be more complicated than that of a direct equation with hydration water of polar side chains. Some specific polar side chains or perhaps combinations of polar side chains of the same or even neighboring protein molecules may be more effective than others.

If we assume that water influenced by the "polar side chains" or "polar-side-chain complexes" has a T_1 of about 40 msec, one can also make a rough estimate as to how much of this water (x) exists in a 20% gelatin solution, assuming that the non-solvent water has the same T_1 as in PEO or PVME solutions at an equal concentration.

$$\frac{1}{1.3} = \frac{(1-0.13-x)}{2.6} + \frac{0.13}{1.2} + \frac{x}{0.04}, \quad (10)$$

$$x = 0.013. \quad (11)$$

PAW were obtained from the p-values for Na citrate and were taken from Ling and Ochsenfeld (1983). Methods of

20	30	40	50	60
80	70	60	50	40
1540	1130	730	480	300
0.34	0.22	0.11	0.05	0.12
66	78	89	95	88
1270	975	670	461	268
1.03×10^{-11}	1.33×10^{-11}	1.93×10^{-11}	2.80×10^{-11}	4.80×10^{-11}

Thus only 1.3% of the water in the 20% gelatin needs to be influenced by the "polar side chains" in order to produce a T_1 of 1300 msec in a 20% gelatin solution.

Now let us turn to living cells. From the AI Hypothesis, the bulk of cell water would be non-solvent to Na citrate; none or very little free water exists in normal resting cells. Ling also expressed the opinion that paramagnetic ions associated with cell proteins most likely contribute to the water proton relaxation (Ling, 1979a, Ling and Tucker, 1980). To discuss NMR properties of water protons in living cells some specific data are needed. We cite the following: On December 26, 1980, four pairs of sartorius muscles from four male frogs were isolated. Using the same instruments the T_1 measured was, for each pair: 580, 600; 610, 610; 680, 690; 620, 640 msec. The mean ± S.D. was 629 ± 38.7 msec. The water contents were all close to 80%.

Let us lump the fast-relaxing water associated with paramagnetic ion-protein complexes and those associated with special "polar side chains" of cell proteins and represent their sum as y with a T_1 equal to .04 sec. Again assuming the bulk of cell water to have the same T_1 as in a 20% PEO or PVME solution (1.3 sec.), we have

$$\frac{1}{0.63} = \frac{0.8-y}{1.3} + \frac{y}{0.04}, \qquad (12)$$

$$y = 0.04. \qquad (13)$$

Thus only 4% of the muscle cell water needs to be associated with paramagnetic ion-protein complex and with special "polar-side chains" to account for the observed T_1 in frog sartorius muscle while the bulk of cell water is "non-solvent." This single set of figures has shown that it is possible to explain at once the NMR data and the low levels of Na^+, sugar, free amino acids, etc., in living cells, without postulating an ever-lengthening list of membrane pumps.

We now turn to another aspect of the findings described in this paper, i.e., the variability of τ_c of the "non-solvent" water and the increase of τ_c with decreasing water content and increasing polymer concentration. We shall demonstrate that this facet of the present finding may help to clarify some other current conflicts of opinions.

The ***Increase of Motional Restriction in the "Non-solvent" Water with Increasing Polymer Concentration as the Basis for Reconciling Seemingly Contradictory Findings of the Dielectric Properties of Cell Water.*** Studies of cells of Artemia cysts revealed a dielectric constant of the cell water distinctly different from that of normal liquid water (Clegg et al, 1982). In contrast, Foster, Schepps, and Schwann (1980) failed to find significant differences in the dielectric constant of water in isolated barnacle muscle at 0°C when compared to normal liquid water.

First we would like to mention that in our experience with barnacle muscles (Reisin and Ling, 1973), the isolation of the muscle cells from the barnacle shell involves inevitable cutting and severe damage of one end of these muscles. This deleterious effect, which tends to spread, seems exacerbated by subsequent exposure to O°C. It would seem that a better alternative is to study frog muscles, which come in intact form and are entirely healthy at O°C. However, even if frog muscles were studied, it would not be altogether surprising that the data do not completely agree with those from the study of Artemia cysts for the following reason.

We have already presented some of the reasons why the bulk of water in all types of living cells would be "non-solvent" to Na citrate (for full account, see Ling, 1983b). Thus if the trend of decreasing τ_c with increasing water content and decreasing polymer (or protein) content (Tables 3 and 4) holds in living cells, then the much higher water contents of barnacle or frog muscle

(80%) will predict a lower τ_c for muscle water protons than for those of Artemia cysts, which had a water content of 50%. Since the Debye rotational correlation time τ_d is equal to 2.5 τ_c, (Eisenberg and Kauzmann, 1967) the anticipated smaller difference in τ_d of normal liquid water may be easily overlooked.

It seems fitting to end this discussion by pointing out that the recent quasi-elastic neutron diffraction studies of Artemia cysts also led to the conclusion that the rotational motional freedom of water in these cells was clearly restricted (Trantham et al, 1983). Rorschach (1983) also studied the neutron diffraction of a 35% solution of PEO, which was in fact a sample from the same PEO used in the present NMR study. He found that the PEO solution strongly resembles the Artemia cyst. From Table 3 one finds that at 35% PEO the estimated τ_c should be about 1.60 X 10^{-11} sec.

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