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The Dynamics of Water in Heterogeneous Systems [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1977 **278**, 59-87
doi: 10.1098/rstb.1977.0031

Phil. Trans. R. Soc. Lond. B. **278**, 59–87 (1977)
Printed in Great Britain

The dynamics of water in heterogeneous systems

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The basic principles of nuclear spin relaxation, dielectric relaxation and quasi-elastic neutron scattering and their use in studying the motions of water molecules are outlined. A summary is given of the time scales associated with the translational and rotational motions of water molecules and of intermolecular proton exchange in pure liquid water. A model is then proposed for the dynamics of water molecules in heterogeneous systems involving regions having differing compositions, water molecules within each region existing in environments both affected by interaction with the macromolecular components and free of their influence and including exchange of water molecules between different environments and regions. The lifetime of the interaction of water molecules with the macromolecular components is assumed long compared with the time for rotation of such bound molecules. Exchange of protons between water molecules and between water molecules and macromolecules is also considered. The ways in which such processes would be expected to affect the observed nuclear magnetic resonance, dielectric and neutron scattering behaviour are outlined. Particular emphasis is placed on nuclear spin relaxation phenomena and the existence and observation of residual dipolar and quadrupolar splittings in the n.m.r. spectra of ^1H and ^2H (D) nuclei in water molecules in such systems, these splittings arising from water molecules dynamically oriented at water/macromolecule interfaces. Details are then given of particular studies of water molecule dynamics in heterogeneous systems using n.m.r., dielectric and neutron scattering techniques. The systems discussed include moist protein powders, protein solutions, phospholipid/water and soap/water mesophases, clay/water systems and biological polymers and tissues. It is concluded that water in these systems is highly mobile, that water molecules affected directly by a macromolecule tumble anisotropically about all axes relative to the macromolecule with correlation times in the region of 10^{-9} s at 260 K and that these molecules exchange with water molecules free of the influence of the macromolecule with a lifetime in the dynamically oriented state of the order of 10^{-6} s at temperatures around 300 K. The ability of nuclear magnetic relaxation studies to distinguish water in different regions of a tissue is discussed and examples are given of the study of the rate of water transport across membranes using these techniques.

1. Introduction

Water pervades all features of the biological world to varying degrees. The study of pure water and aqueous solutions of simple molecules and ions is still far from being a completed story although considerable advances in understanding their properties have been made in the last decade (Franks 1972; Kavanau 1964; Eisenberg & Kauzmann 1969). The importance to biological and medical sciences of a detailed knowledge of the nature of water in cells, tissues, membranes, etc. is difficult to assess. It is a fact that many of the major advances in understanding the nature of biological structures and processes have tended to be concerned with the non-aqueous components, the water being regarded, if considered at all, as an ever present medium in which the structures exist and the processes occur. Yet it is clear that, in general, living systems only retain their biological function in the presence of, amongst other things, water. In addition an aqueous medium is virtually always the means of transporting material into,

around and through living systems and of maintaining many essential physical properties. It would seem therefore that an appreciation of the nature of water in biological systems is of considerable relevance, water being a necessary and integral part of the whole complex of life processes.

In this paper we shall investigate some aspects of the dynamics of water in heterogeneous systems, particularly, but not exclusively, in systems of biological origin. We shall take the term dynamics to include all motions other than coherent intra- or intermolecular vibrations. Thus we shall be concerned largely with the chaotic reorientation and translation of water molecules. In the latter case we shall also be concerned with the interchange of hydrogen atoms between different water molecules. The study of these motions in water has mainly been carried out by using three techniques: nuclear magnetic resonance and relaxation, dielectric relaxation and quasi-elastic neutron scattering. The basic principles of these techniques are outlined in the next section.

2. Techniques

(*a*) *Nuclear magnetic resonance and relaxation*

A nucleus with a non-zero spin angular momentum, characterized by a spin quantum number I, has a magnetic moment and, if $I > 1$, an electric quadrupole moment. The nuclei relevant to studies of water are $^1H(I = \frac{1}{2})$, $^2H(I = 1)$ and $^{17}O(I = \frac{5}{2})$. In the presence of a large magnetic field $\boldsymbol{B}_0$ a magnetic nucleus precesses at a frequency ω_0 $(= -\gamma \boldsymbol{B}_0$, γ being characteristic of the isotope concerned) and has $(2I+1)$ energy states available to it. The resonance frequency, ω_0, actually depends on its electronic environment. However, in this paper we shall not be concerned with such chemical shift effects and so will not discuss this further.

A fluid molecular system containing a large number of magnetic nuclei eventually reaches a state of thermal equilibrium when in the field $\boldsymbol{B}_0$, this state being characterized by a Boltzmann distribution of the nuclei between the $(2I+1)$ energy states with the temperature of this distribution corresponding to the temperature of the molecular degrees of freedom (i.e. translation, rotation etc.) which are collectively referred to as the lattice. This process of energy exchange between the magnetic energy of the nuclei and the lattice is known as spin-lattice relaxation and the time constants characterizing it are usually designated T_1. The unequal thermal equilibrium populations of the $(2I+1)$ energy states leads to a net macroscopic equilibrium nuclear magnetization, $\boldsymbol{M}_0$, along the field $\boldsymbol{B}_0$.

At equilibrium in $\boldsymbol{B}_0$ the precessing nuclear magnets have random phases and there is no net transverse nuclear magnetization (i.e. magnetization perpendicular to $\boldsymbol{B}_0$). However, application of a circularly polarized magnetic field $\boldsymbol{B}_1(\omega)$ in the transverse plane can produce a macroscopic transverse magnetization when $\omega \approx \omega_0$. On removal of the resonant field, $\boldsymbol{B}_1(\omega)$, this magnetization decays to zero, the time scale of this decay being characterized by one or more time constants designated T_2. This process, known as transverse relaxation, is one of dephasing of the precessing nuclear spins. Among others, a further relaxation process, characterized by a time constant T_{1_ρ}, may be studied. This process corresponds to the decay of nuclear magnetization aligned along $\boldsymbol{B}_1(\omega)$ rather than $\boldsymbol{B}_0$ and is known as spin-lattice relaxation in the rotating frame.

Relaxation of nuclear magnetization arises from the local interactions experienced by the nuclei. For studies of water, the two most important interactions are the nuclear magnetic dipole–dipole coupling and, for ^2H and ^{17}O, the nuclear electric quadrupole coupling. The

former, which is of most importance for protons, arises from the local dipolar magnetic fields at the site of one nucleus due to its neighbours, while the latter is due to the coupling of the nuclear electric quadrupole moment (which has a fixed spatial relation to the nuclear magnetic moment) to the electric field gradients arising from the surrounding electrons. It is clear that in a fluid system these interactions are going to be chaotically fluctuating as the molecules containing the nuclei tumble and diffuse. The efficiency of these local interactions in causing nuclear magnetic relaxation depends on the nature and time scales of the fluctuations and this is the basis of the use of this technique for studying molecular motion. Because of the complexity of fluctuations in a many particle system the time dependence of the local interactions are expressed in terms of so-called time correlation functions. For example, the correlation function, $G(\tau)$, for a fluctuating local field, $\boldsymbol{b}_l(t)$, could be expressed as

$$G(\tau) = \langle \boldsymbol{b}_l(t) \,.\, \boldsymbol{b}_l(t+\tau) \rangle = \langle b_l^2 \rangle \exp - |\tau/\tau_c|, \tag{1}$$

where the angular brackets imply an average over all possible situations in the system and where the second equality is an often made assumption as to the form of $G(\tau)$. This introduces the so-called correlation time, τ_c, which characterizes the time scale of the fluctuations. Whether or not an exponential form for $G(\tau)$ and a single τ_c are satisfactory assumptions can only be determined by the degree of agreement with experiment and by sensible evaluation of each particular situation. In many cases this simple model is inadequate and several correlation times (Woessner 1962) or even a continuous distribution of correlation times (Resing 1965) must be introduced.

The nuclear magnetic relaxation rates are related to the time correlation functions of the fluctuating local interactions by Fourier transformation. What this amounts to is that the fluctuations, say in $\boldsymbol{b}_l(t)$, are frequency analysed. This gives rise to the so-called spectral density or power spectrum, $J(\omega)$, of the fluctuations. For the $G(\tau)$ given in equation (1) this has the form

$$J(\omega) = \langle b_l^2 \rangle \frac{2\tau_c}{(1+\omega^2\tau_c^2)} \tag{2}$$

which indicates that the available strength of the interaction, $\langle b_l^2 \rangle$, is spread evenly over all frequencies up to τ_c^{-1} but that very little of the interaction is available at higher frequencies.

For magnetic dipole–dipole interactions between like spins the general form of the relaxation rates are

$$(T_1)_d^{-1} = \tfrac{2}{3}\overline{\Omega_d^2}[j(\omega_0) + 4j(2\omega_0)], \tag{3}$$

$$(T_2)_d^{-1} = \tfrac{2}{3}\overline{\Omega_d^2}[\tfrac{3}{2}j(0) + \tfrac{5}{2}j(\omega_0) + j(2\omega_0)], \tag{4}$$

$$(T_{1\rho})_d^{-1} = \tfrac{2}{3}\overline{\Omega_d^2}[\tfrac{3}{2}j(2\omega_1) + \tfrac{5}{2}j(\omega_0) + j(2\omega_0)], \tag{5}$$

where $j(\omega)$ is a 'reduced' spectral density, which for the form given in equation (2) would be $[\frac{1}{2}J(\omega)/\langle b_l^2 \rangle]$, and Ω_d^2 $(= \gamma^2 \langle b_l^2 \rangle)$ is a measure of the appropriate mean-squared dipolar coupling strength.

For a spin $I = 1$ nucleus the relaxation rates arising from fluctuating electric quadrupole interactions are as follows

$$(T_1)_q^{-1} = \frac{\overline{\Omega_q^2}}{160}[6j(\omega_0) + 24j(2\omega_0)], \tag{6}$$

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$$(T_2)_q^{-1} = \frac{\overline{\Omega_q^2}}{160}[9j(0) + 15j(\omega_0) + 6j(2\omega_0)], \tag{7}$$

$$(T_{1\rho})_q^{-1} = \frac{\overline{\Omega_q^2}}{160}[9j(2\omega_1) + 15j(\omega_0) + 6j(2\omega_0)], \tag{8}$$

where $\overline{\Omega_q^2}$ is the mean squared electric quadrupole coupling energy in angular frequency units and the $j(\omega)$ are reduced spectral densities similar in form to those given above.

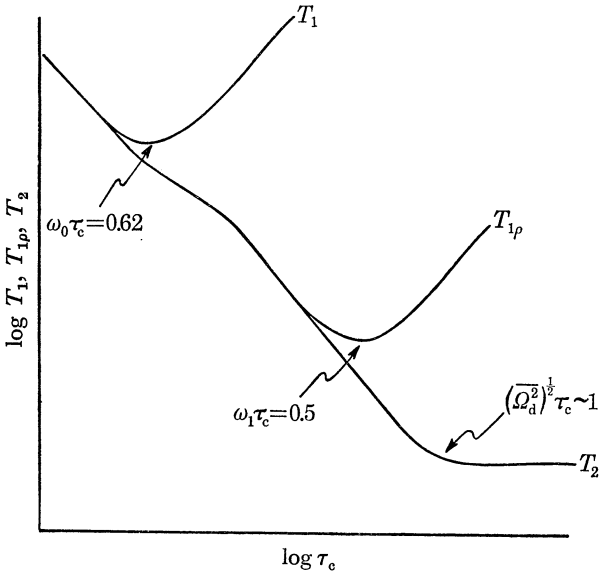

FIGURE 1. Variation of T_1, $T_{1\rho}$ and T_2 with correlation time τ_c for a system described by equations (2)–(5) in the text. Note that for $\omega_0\tau_c \ll 1$ all relaxation times are equal and that minima in T_1 and $T_{1\rho}$ occur when $\omega_0\tau_c \approx 0.62$ and $\omega_1\tau_c = 0.5$ respectively. T_2 becomes independent of τ_c when $(\overline{\Omega_d^2})^{\frac{1}{2}}\tau_c > 1$.

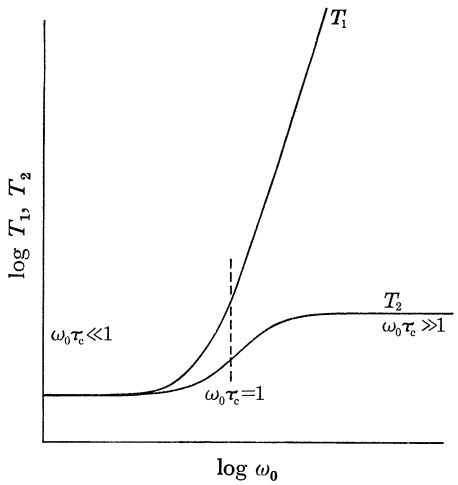

FIGURE 2. Variation of T_1 and T_2 with resonance frequency ω_0 for a system described by a single correlation time, isotropic motion model (equations (2)–(5) in text). Note that when $\omega_0\tau_c \ll 1$ $T_1 = T_2$ and both are independent of frequency whereas when $\omega_0\tau_c \gg 1$, $T_2 \ll T_1$ and T_1 is proportional to ω_0^2. T_2 is only dependent on frequency in the range $0.1 < \omega_0\tau_c < 10$.

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The dependence of T_1, T_2 and $T_{1\rho}$ on τ_c, ω_0 and ω_1 is illustrated for a simple single correlation time isotropic motion model in figures 1 and 2.

For more detailed accounts of nuclear magnetic relaxation the reader is referred to books by Abragam (1961), Slichter (1963) and Farrar & Becker (1971).

Nuclear magnetic relaxation processes may be studied in a number of ways but the method of most general applicability involves excitation of the nuclei by means of pulses of the radiation field, $\boldsymbol{B}_1(\omega)$. These pulses are characterized by, among other things, the angle through which they turn the macroscopic magnetization vector, $\boldsymbol{M}$. For example, a $90^\circ_{x'}$ pulse applied to a spin system at thermal equilibrium turns $\boldsymbol{M}_0$ from the $\boldsymbol{B}_0$ direction and places it in the transverse plane, along the y' axis in fact. x' and y' refer to a set of axes, in the plane perpendicular to $\boldsymbol{B}_0$, rotating at the frequency ω. Various combinations of pulses are used to study the different relaxation processes (Farrar & Becker 1971).

(*b*) *Dielectric relaxation*

Any molecule or part of a molecule which has an electric dipole moment will tend to be oriented in an electric field. If the field is made to alternate then, when the alternation frequency becomes comparable to the rate at which the dipole can reorient, absorption of energy from the field rises to a maximum and the electrical permittivity decreases. This process is expressed in terms of a complex permittivity, ϵ^*, given by

$$\epsilon^* = \epsilon' - \mathrm{i}\epsilon'', \tag{9}$$

where ϵ'' is the dielectric absorption and ϵ' the dielectric 'constant'.

For a dipole whose reorientation is characterized by a single correlation time τ_ϵ, ϵ' and ϵ'' are given by

$$\epsilon' = \epsilon_\infty + \frac{(\epsilon_s - \epsilon_\infty)}{1 + (\omega\tau_\epsilon)^2}, \tag{10}$$

$$\epsilon'' = \frac{(\epsilon_s - \epsilon_\infty)\omega\tau_\epsilon}{1 + (\omega\tau_\epsilon)^2}, \tag{11}$$

where ϵ_s and ϵ_∞ are the low- and high-frequency limits of ϵ^*.

As with nuclear magnetic relaxation a distribution of reorientation times is sometimes indicated by the experimental results. In such a case the results are often represented by the so-called Cole–Cole equation (Cole & Cole 1941)

$$\epsilon^* = \epsilon_\infty + \frac{(\epsilon_s - \epsilon_\infty)}{1 + (\mathrm{i}\omega\tau_\epsilon)^{(1-\alpha)}} \tag{12}$$

for which a graph of ϵ'' against ϵ' has been shown to be a semi-circle with its diameter making an angle $(\frac{1}{2}\alpha\pi)$ with the ϵ' axis. Other forms of equation employing different distributions of relaxation times may be used (Grant & Sheppard 1974). The relationship between τ_ϵ and τ_r, the correlation time for reorientation obtained from nuclear spin relaxation measurements, depends on the model used for the reorientation process (Hertz 1967).

(*c*) *Neutron inelastic scattering*

A monoenergetic beam of slow neutrons passing through a material is scattered by interactions with the nuclei, protons being particularly effective in this respect. If the nuclei causing the scattering are moving then the scattered neutrons experience changes in energy and

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momentum. These can be thought of as being of two types; inelastic events in which quantized energy changes occur involving discrete rotational, torsional, vibrational levels associated with the scattering centres and, quasi-elastic events giving rise to Doppler shifts having their origin in the chaotic translational/rotational displacements of the scattering centres. These two types of scattering are analogous in many respects to Raman and Rayleigh scattering in optical spectroscopy. The important feature of neutron scattering from our point of view is that, for quasi-elastic scattering from scattering centres undergoing continuous Fick's law diffusional motion, it has been shown that the width, ΔE, of the quasi-elastic peak (centred about zero energy transfer) is given by (Stirling & White 1971)

$$\Delta E = 2D_s\hbar Q^2$$

where D_s is the self-diffusion coefficient of the scattering centres and Q is the momentum transfer which has the form

$$Q \sim (4\pi/\lambda) \sin \tfrac{1}{2}\theta,$$

in which θ is the scattering angle and λ the incident neutron wavelength.

3. Dynamics in liquid water

In this section we briefly summarize the time scales in liquid water of the various motions of concern to us in this paper. Figure 3 illustrates the processes we are interested in.

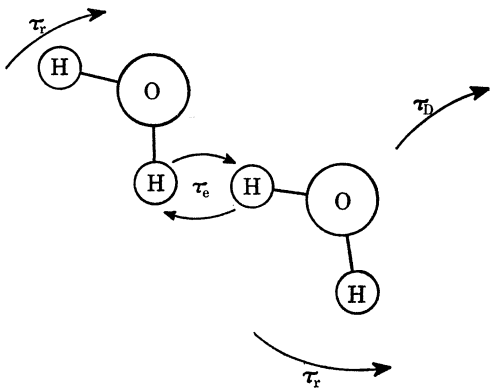

Figure 3. Dynamic processes in water. τ_D is a correlation time for translational diffusion ($\sim d^2/6D_s$, where d is a jump distance and D_s the self-diffusion coefficient); τ_r, a correlation time for reorientation and τ_e, the lifetime of a hydrogen atom in a water molecule between hydrogen exchange events.

(a) *Translational diffusion*

Self-diffusion in liquid water has been studied by pulsed n.m.r. spin-echo techniques (Gillen, Douglass & Hoch 1972), neutron scattering (White 1971*a*, *b*) and radio-tracer methods (Mills 1971). Although there are some differences in the values of D_s obtained with these techniques, at 298 K it is close to 2.2×10^{-9} m^2 s^{-1}. The reasonable agreement between the tracer and n.m.r. measurements on the one hand and the neutron scattering measurements on the other is, at first sight, a little surprising since the time scales of observation of the two groups of techniques are quite different. Neutron scattering effectively observes the motion of the water protons over times of the order of 10^{-14}–10^{-11} whereas the other techniques involve observation times greater than 10^{-3} s. In neutron scattering it might have been expected that the effects of rotational

ions would have been inextricably bound up with the effects of centre-of-mass displacements. However, this appears not to be so for water and this is the basis for the use of neutron ttering for the study of self-diffusion in water over short time scales and distances (White 1 *a*, *b*).

f a random-walk model is assumed for the self-diffusion process with τ_D being the time ween displacements d, then these quantities are related by

$$d^2 = 6D_s\tau_D. \tag{13}$$

is taken to be of the order of 2.5×10^{-10} m then τ_D is of the order of 3×10^{-12} s.

Ve conclude this section by noting that the temperature dependence of D_s indicates an vation energy varying from about 12 kJ mol^{-1} at 473 K to around 46 kJ mol^{-1} near 243 K. ese facts have been interpreted in terms of a diffusion process which involves the breaking n increasing number of hydrogen bonds at low temperatures (Gillen *et al.* 1972; O'Reilly, erson & Scheie 1973).

(*b*) *Rotational motion*

Dielectric relaxation studies (Grant & Sheppard 1974; Hasted 1972) and spin-lattice relation measurements on ^{2}H and ^{17}O nuclei (Hindman, Zielen, Svirmickas & Wood 1971) lead to the result that around 290 K the reorientational correlation times of the water ecule (τ_e, τ_r) are of the order of 10^{-12}–10^{-11} s. The precise values depend on the models sen for the motion but it can be seen that it is of the same order of magnitude as τ_D. The perature dependence of the ^{1}H, ^{2}H and ^{17}O spin-lattice relaxation times in water has been rpreted in terms of a mechanism involving the rotational diffusion of a monomeric species quilibrium with a so-called lattice species (Hindman *et al.* 1971; Hindman, Svirmickas & od 1973). More recently, however, the dielectric measurements have been interpreted in ns of two relaxation times, differing by a factor of two (Grant & Sheppard 1974). O'Reilly *et al.* (1973) have used a model involving the ice VII–ice I c structures as a basis for interpreting temperature dependence of several transport properties of liquid water.

n the physiological temperature region the activation energy for reorientation is of an order ch, independent of detailed models, implies the breaking of at least one hydrogen bond in rate determining step.

(*c*) *Intermolecular proton exchange*

Exchange of protons between water molecules occurs with a lifetime, τ_e, of the order of 10^{-3} s. s was originally studied by n.m.r. (Meiboom 1961) via measurements of $T_{1\rho}$ and T_2 for the tons in water slightly enriched with ^{17}O. The technique relies on the fact that, when bonded a ^{17}O atom, the proton experiences a scalar spin–spin coupling which the exchange makes e dependent. Meiboom studied the pH dependence of the exchange process and found that average lifetime between exchanges, τ_e, could be represented by

$$(\tau_e)^{-1} = \tfrac{2}{3}k_1[H^+] + k_2(k_w/[H^+]) \tag{14}$$

h $k_1 = (10.6 \pm 4) \times 10^9$ l mol^{-1} s^{-1} and $k_2 = (3.8 \pm 1.5) \times 10^9$ l mol^{-1} s^{-1}. Thus at pH 7, = 0.9 ms.

Knispel & Pintar (1975) have recently reported some measurements of τ_e in water at various peratures using two pulsed n.m.r. techniques. In one they measured $T_{1\rho}$ as a function of the ength of the field $\boldsymbol{B}_1(\omega)$ (expressed as a frequency $\omega_1 = \gamma \boldsymbol{B}_1$) and obtained a dispersion. This ses when $\omega_1\tau_c$ is of the order of unity (see equations (2) and (5)) and allows a direct measure

of both τ_c and the interaction strength modulated by the exchange process. The second technique is very similar. T_2 is measured by means of a multiple-pulse sequence (Carr & Purcell 1954; Meiboom & Gill 1958). As with the $T_{1\rho}$ measurements a dispersion may be observed in T_2 as a function of t_p, the pulse separation. This occurs when $t_p \approx \tau_e$ (Gutowsky, Vold & Wells 1965). Examples of the T_2 dispersions obtained by Knispel & Pintar (1975) are shown in figure 4.

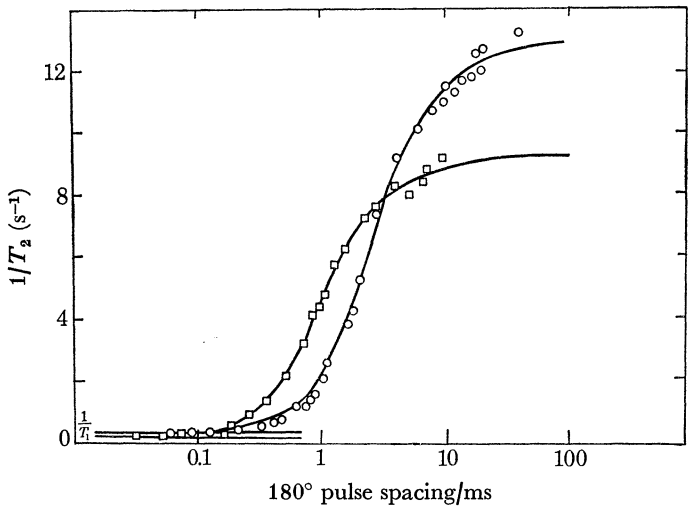

FIGURE 4. The dependence on 180° pulse spacing of the proton transverse relaxation rate in ^{17}O enriched water as measured by a Carr–Purcell/Meiboom–Gill spin echo pulse sequence (Knispel & Pintar 1975). ○, 296 K, $\tau_e = 0.71$ ms; □, 331 K, $\tau_e = 0.28$ ms.

(*d*) *Effects of solutes*

Effects of small solutes on the motions of water molecules in dilute aqueous solutions are varied, both increases and decreases in the rates of rotation and diffusion being observed. The important point for our purposes however is that the changes produced by solute concentrations comparable to the concentrations of small solutes in biological materials are negligible in comparison with those produced by macromolecules. As we shall see this difference is largely a result of the time scales of the motions of the solutes and solute–water complexes.

4. HETEROGENEOUS SYSTEMS

(*a*) *General characteristics*

Most systems of biological relevance from moist protein powders, protein solutions, phospholipid/water dispersions etc. through to whole tissues and real membranes are heterogeneous. By this we imply that they contain molecules of quite different sizes, that these may be organized in a non-random manner in space and that water and ions may be distributed throughout the system in a non-uniform way. In figures 5 and 6 we illustrate schematically some of the features of heterogeneous materials which are relevant to the study of the properties of water in such systems.

Figure 5 illustrates the fact that in many biological materials there exist regions which have different chemical compositions, e.g. water contents. Striated muscle is a typical case in which

we can identify, for example, inter- and intracellular regions as having quite different compositions. The intracellular region itself also has distinct areas of differing composition, the various regions of the sarcomere containing thin filaments only, thick filaments only and the overlap region containing both types of muscle protein serving as examples. In many cases the water molecules are free to move from one region to another and their lifetime in a given region will reflect the geometry and dimensions of the region and the effective self-diffusion coefficient of the water molecules in each region. If barriers to this movement exist, such as a cell membrane, then the lifetime of a water molecule in a particular region may be partly or wholly determined by this barrier.

In figure 6 the situation is examined from a more microscopic viewpoint. The macromolecular components of our heterogeneous system are illustrated as providing interfacial regions

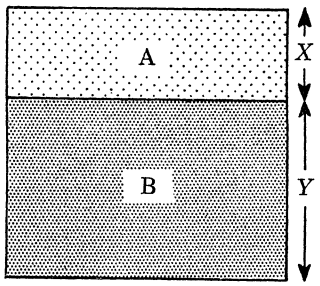

FIGURE 5. A schematic illustration of large scale heterogeneity. Distinct regions of a material (e.g. A and B) may have differing compositions (e.g. water contents), geometries and dimensions (e.g. X, Y, etc.) and may also differ in such things as degrees of order and anisotropy in internal structure. Water molecules may transfer between regions at rates which may reflect both the effective self-diffusion coefficients for water within each region and any barrier function provided by the interface between the regions.

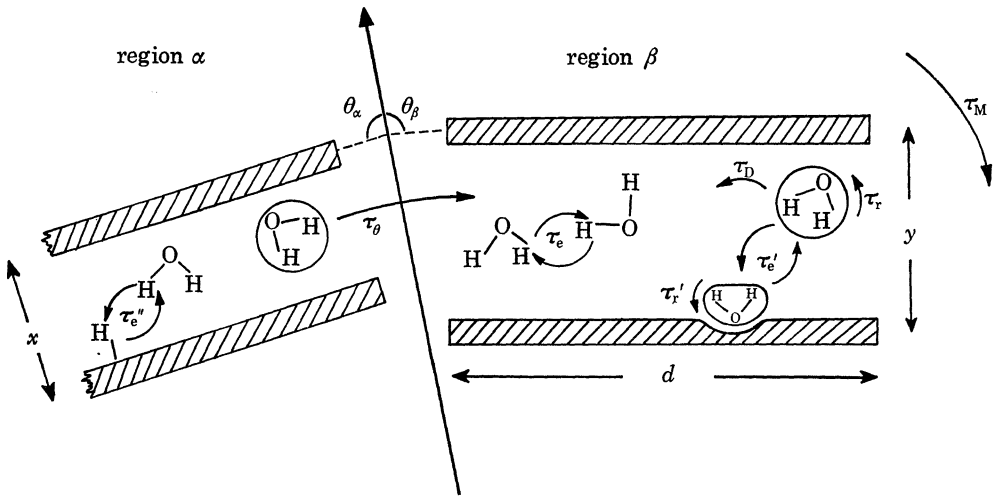

FIGURE 6. A schematic illustration of small scale heterogeneity and various dynamic processes which may be experienced by water molecules in such a system. The shaded regions represent macromolecular structures characterized by dimensions d, x, y etc., orientations θ_α, θ_β etc. with respect to an external fixed axis, and correlation times for tumbling, τ_m. Water molecules free of the influence of the macromolecules diffuse and rotate and exchange protons with characteristic times τ_D, τ_r and τ_e respectively. Water molecules interacting with the macromolecule tumble anisotropically, this process being represented by a collective correlation time τ_r', and have a lifetime in this state designated by τ_e'. Water molecules may diffuse from one region to another, their lifetime in a given region being $\tau_\theta (\sim d^2/2D_s)$ whilst they exchange protons with macromolecules with a lifetime τ_e''.

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which on some distance and time scales, d and τ_m, have a particular orientation with respect to some external reference axis. (In figure 6 two regions, α and β, are shown having orientations θ_α and θ_β.) The distance d is often much larger than the size of a water molecule and, in systems where the macromolecules concerned are structural materials such as collagen, τ_m can be very large or even infinite. For solutions of proteins, on the other hand, τ_m will reflect the tumbling of the protein molecule in part or as a whole.

Figure 6 shows the water as existing between the interfacial regions, the distances between adjacent interfaces being designated as x and y. The water molecules can undergo a variety of motions in such a system as illustrated.

Within a given region (i.e. α or β) a water molecule can either be interacting with the macromolecule or not. How far from the macromolecule a water molecule must be for it to be regarded as non-interacting is not entirely clear. It has been suggested that long range effects on water structure and dynamics exist in this type of situation (Ling 1962). The bulk of experimental evidence available, as we shall see, is more consistent with local effects involving not much more than a layer or two of water molecules (Stirling & White 1971; Olejnik & White 1972; Hayter, Hecht, White & Tiddy 1974), and in the following discussion we adopt this point of view. It is probable that water molecules permeate the macromolecular regions and that they can pass from the predominantly aqueous medium into and through the macromolecular structure. As yet there is little experimental evidence relevant to this so we shall regard the macromolecular components as providing impermeable interfaces although it is clear that proteins do contain water as an integral part of their structure and that this water is not uniformly distributed throughout.

A water molecule which is free of the direct influence of the macromolecular interface will tumble, diffuse and exchange protons in much the same way as a similar molecule in a dilute aqueous solution containing the appropriate concentrations of whatever small molecules and ions are present. In figure 6 these motional processes are represented by the correlation times τ_r, τ_D and τ_e which, in general, will not depend strongly on variations in region (i.e. whether we are considering region α or β etc.). At any instant, some fraction of the water molecules in the system will be interacting with the macromolecular surface. These interactions could be of many types, arising from specific ion-dipole forces, dispersion forces, hydrophobic effects on water structure etc. Whatever their source, such interactions are bound to be anisotropic in so far as the water molecule is concerned. What this implies is that such water molecules move in an anisotropic potential, the spatial properties of which (referred to an external fixed axis system) remain unchanged on the time scale of many reorientations of the water molecules. This difference of time scale is crucial, particularly for the observation of these various motions via nuclear magnetic resonance. In bulk liquid water, for example, the instantaneous potential which a molecule experiences is likely to be anisotropic, but the axes defining this anisotropy change direction as fast if not faster than the rate at which individual molecules reorient. A water molecule at the macromolecular interface then, reorients at different rates about three Cartesian axes fixed in the macromolecular surface. These motions are indicated in figure 6 by the collective correlation time τ_r'. Such an anisotropically tumbling molecule remains 'bound' to the macromolecule until a thermal fluctuation gives it enough energy to interchange its situation with a nearby 'free' water molecule. This process is shown in figure 6 to be associated with a lifetime τ_e'. Another process which may be relevant is the diffusion of water molecules between adjacent regions. The correlation time associated with this is shown in figure 6 as τ_θ

and is the time taken for a water molecule to diffuse a distance of the order of d in the appropriate direction. This depends on the effective diffusion coefficient of the water molecules in the regions involved. Finally, another dynamic process which can occur is the exchange of protons on the macromolecule (e.g. –OH, –NH etc.) with water protons. This is represented by a lifetime τ''_e.

Before describing in detail examples which illustrate the study of some of these processes in heterogeneous systems we outline the ways in which each of the three techniques mentioned earlier might be expected to reflect the features of heterogeneous systems contained in figures 5 and 6. Of these the first, nuclear magnetic resonance, probably gives the most detailed information.

(*b*) *Nuclear magnetic resonance*

The effects observable in the magnetic resonance properties of nuclei of water molecules in a particular heterogeneous system depends on which nucleus is studied and on the relative time scales of the various processes discussed above. An important general principle we shall be concerned with is the effect of exchange of nuclei between sites in which they are characterized by well defined n.m.r. parameters such as resonance frequencies and relaxation times. In qualitative terms, if the rates of exchange are slow compared with the site relaxation rates and the differences in site resonance frequencies then each site gives rise to its own characteristic resonance signal. On the other hand, if the rates of exchange are fast then only an average resonance frequency and average relaxation rates are observed. As we shall see, it is possible to obtain information on the time scales of the various processes from a study of the nature of the resonance signals and their relaxation behaviour. In considering the effects we might expect to observe we shall make certain assumptions as to the relative time scales of the processes involved based on the results of some of the studies we shall discuss later.

We start by considering a water molecule interacting with some site on a macromolecule (see figure 6). We suppose that its average lifetime in this site, τ'_e, is considerably longer than the correlation times, τ'_r, for its reorientation in this site. For the moment we also assume, for simplicity, that the nuclei in this water molecule do not experience any significant interactions with nuclei in other water molecules or in the macromolecule. We also do not consider the possibility of the binding site involving an electronically paramagnetic metal ion. The study of this latter situation is a subject in itself and can lead to information concerning the number of water molecules bound to the ion and often to information concerning their dynamics (Dwek 1973). If the nuclei in the water molecule we are considering are protons then, because the proton–proton vector in the molecule is tumbling anisotropically and, hence, has a non-spherically symmetric distribution in space, the dipole–dipole coupling between the two protons does not average to zero as it would do in a water molecule in the isotropic liquid. This results in a splitting of the proton resonance into a doublet, the frequency separation of which, Γ_d, is a direct measure of the dipole–dipole coupling averaged over the non-spherical motion of the molecule (Johansson & Drakenberg 1971). This splitting depends on the angle θ between the macromolecular surface and the external field $\boldsymbol{B}_0$. The (major) part of the dipolar interaction, which to first order is averaged to zero by the anisotropic tumbling motion, contributes to the relaxation of these protons according to relationships such as equations (3), (4) and (5). We now assume that the effect of the intermolecular proton dipolar interactions is to give an additional contribution to the relaxation rates and not to produce any further splittings of the resonance lines.

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In the same anisotropic situation, ^2H and ^{17}O nuclei in a water molecule will experience non-zero average electric quadrupole interactions (Johansson & Drakenberg 1971; Wennerström, Lindblom & Lindman 1974) which, in the case of the ^2H nucleus ($I = 1$) would result in a doublet spectrum with frequency separation Γ_q. The (major) part of the quadrupolar interaction averaged by the tumbling of the water molecule would, as in the case of the proton dipolar interaction, determine the relaxation times, T_{ib} ($i = 1, 1_\rho, 2$; b = bound site). We should also note that, relative to a water molecule remote from the macromolecular surface, nuclei in the bound water molecules may experience a resonance frequency shift due to (i) electronic changes produced by the interaction with the macromolecule and (ii) bulk magnetic susceptibility differences between the macromolecular structure and the aqueous medium (Glasel & Lee 1974; Packer 1973). Unlike the dipolar and quadrupolar interactions these two effects depend on the strength of the field $\boldsymbol{B}_0$ and, in principle, could be distinguished on this basis.

In a complex heterogeneous system each region (i.e. α and β in figure 6) will probably contain a number of different types of binding sites giving rise to different values of the various effects (splittings, shifts, correlation times, relaxation times) mentioned above. If τ_e' for each such site were sufficiently large then the observed n.m.r. signal would be a superposition of signals from free and all bound sites. On the basis of experience however, we shall only consider the situation in which τ_e' and p_b (the fraction of nuclei in bound sites) are sufficiently small to ensure that fast exchange conditions apply (i.e. $\tau_e' \ll T_{it,b}$, $\Delta\omega$; f $\equiv$ free, $\Delta\omega \equiv$ any frequency differences modulated by the exchange process). Under these conditions only average splittings and relaxation times are observed and for a single region assuming only a single type of binding site these are

$$\omega_{d,q} = p_b \Gamma_{d,q}, \tag{15}$$

since the splittings are zero in the free state and, if $\tau_e' \ll T_{ib}$ (Zimmerman & Brittin 1957)

$$(T_i)^{-1} = (p_f/T_{if}) + (p_b/T_{ib}). \tag{16}$$

However, in considering the relaxation rates we must also allow for the fact that the exchange process itself can provide an additional relaxation contribution because it causes the nuclei to experience time dependent splittings and resonance frequency shifts. As we discussed above in connection with proton exchange in bulk water (§3*c*), measurements of T_{1_ρ} as a function of ω_1 and T_2 as a function of t_p can be used to study processes with rather long correlation times. We might expect then, that if $\omega_1\tau_e'$ and/or (t_p/τ_e') can be made of the order of unity, dispersions in T_{1_ρ} and T_2, similar to those illustrated in figure 4 will be observable leading to direct measurements of τ_e' and information concerning p_b and $\Gamma_{d,q}$.

If the system under study contains regions having different orientations (θ_α, θ_β, etc.) with respect to the field $\boldsymbol{B}_0$ then a distribution $P(\omega_{d,q})$ of splittings would exist. If, in addition, the dimensions of the regions, d, are such that $\tau_\theta (\approx d^2/2D_s) < \omega_{d,q}^{-1}$, then further averaging of the $\omega_{d,q}$ values will occur. Only if $P(\omega_{d,q})$ corresponds to a spherically symmetric distribution of angles θ will this diffusional average tend to zero in the fast exchange limit ($\tau_\theta \omega_{d,q} \ll 1$).

For protons, intermolecular exchange can also lead to averaging to zero of ω_d. This is because ω_d arises from an intramolecular two-spin coupling, the intermolecular interactions being completely averaged. Again, measurements of T_{1_ρ} and T_2 could reveal information on the timescales of these diffusional and exchange averaging processes and the interaction strengths

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involved. It is also possible to use a combination of two two-pulse n.m.r. sequences to study these processes (Woessner 1975). This approach is very similar to that involving $T_{1\rho}$ and T_2 measurements.

Referring to figure 5 we should note that all of the processes and phenomena described above may be occurring in each of the regions A, B, etc. If the dimensions of these regions are such that slow exchange conditions hold, then the observed nuclear relaxation behaviour will be a superposition of components reflecting the properties of each region. The resolution of these components and the complete evaluation of their individual characteristics is a daunting experimental task which has yet to be achieved for any natural system.

Before leaving this survey of n.m.r. in heterogeneous systems we should note that most biological systems contain protons other than those in water. In many cases the relaxation characteristics of these non-aqueous protons are sufficiently different from those of the water protons to allow easy separation of the two or their relative proportions are low enough for them to be ignored. Use of ^{2}H substitution and ^{17}O enrichment techniques help in the resolution of any such problems.

(*c*) *Dielectric relaxation*

Dielectric relaxation effectively gives an equilibrium picture of the water molecules in a system. For example, molecules in a bound site in which they have a certain reorientation rate give a contribution to ϵ^* characteristic of their properties in that site. Thus, in principle for the bound water molecules in a heterogeneous system, a dispersion will occur in ϵ' at a frequency ω where $\omega\tau_r'$ is of the order of unity. Free water molecules will also give rise to a dispersion at appropriate, higher frequencies. Dielectric studies of aqueous heterogeneous systems are, however, subject to some complications arising from contributions to the frequency dependence of ϵ^* from other than dipolar orientation effects (de Backer & Watillon 1973; de Loor 1968). These are due to conductivity, electrical double layers at interfaces etc., and become more important at low frequencies. Above 10^9 s^{-1} they are not, in general, particularly significant but in the important range 10^7–10^9 s^{-1} care must be taken to correct for these effects if reorientation frequencies are to be unambiguously identified.

(*d*) *Neutron quasi-elastic scattering*

The study of water dynamics in biological systems by neutron scattering is complicated by the presence of non-aqueous protons. However, the motions of these may often be on a different time scale from those in the water molecules and under these circumstances or in systems with high water contents or low proton-content non-aqueous components, neutron scattering can give direct information on the short-time diffusional behaviour of the water protons. Like dielectric relaxation, neutron scattering gives a superposition of information from different sites although the results are generally interpreted in terms of a single diffusion coefficient.

5. Studies of water dynamics in heterogeneous systems

We now examine some examples of studies of water dynamics in heterogeneous systems which are illustrative of the state of knowledge in this area. It is not intended to provide a comprehensive review but to indicate, where possible, the time scales of the various processes, described in the previous section, in different systems. Two reviews of interest in this context are those by Cooke & Kuntz (1974) and Kuntz & Kauzmann (1974).

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Before dealing with particular examples we should note that a great deal of evidence, some of which is admittedly somewhat circumstantial, supports the view that a fraction of the water in heterogeneous and biological systems has properties quite distinct from those of bulk water. For example, it is a well established fact that in such systems as solutions of polysaccharides (Duff & Derbyshire 1975) and proteins (Kuntz 1971; Kuntz, Brassfield, Law & Purcell 1969) and in tissues (Duff & Derbyshire 1974; Belton, Packer & Sellwood 1973; Fung, Durham & Wassil 1975) a fraction of the water, amounting to approximately 0.3–0.6 g water/g non-aqueous component, does not freeze and remains unfrozen to temperatures below 200 K. More detailed discussions of this and related evidence are given in the reviews mentioned above (Cooke & Kuntz 1974; Kuntz & Kauzmann 1974) and below.

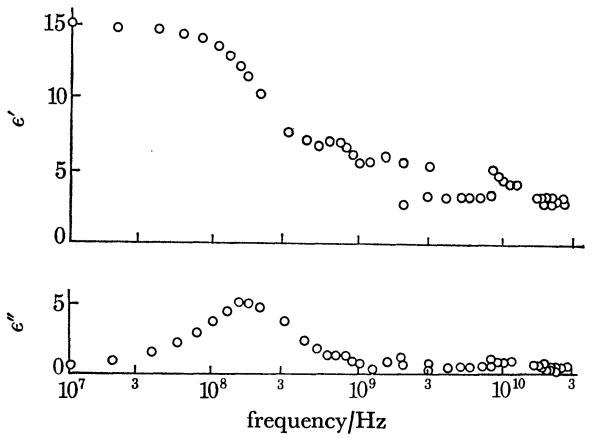

FIGURE 7. The variation with frequency of the dielectric constant (ϵ') and loss (ϵ'') for lysozyme containing 0.34 g water/g protein at 298 K. A distinct dispersion and a loss maximum occur at a frequency of $\sim 2 \times 10^8$ s^{-1} corresponding to a dielectric relaxation time of 10^{-9} s (Harvey & Hoekstra 1972).

(*a*) *Simple protein/water systems*

The figure of 0.3 g water/g protein appears to correspond to a discontinuity in the dielectric properties of the lysozyme/water system studied by Harvey & Hoekstra (1972). They monitored ϵ' and ϵ'' at 25 GHz (close to the dispersion frequency for bulk liquid water) as a function of water content and only observed a significant increase in these quantities above this critical water content. This was interpreted as indicating that, only above this concentration, did water molecules having properties similar to bulk liquid water appear. These workers also measured the frequency dependence of ϵ' and ϵ'' for this system at two water contents, 0.34 and 0.54 g water/g lysozyme. Their results, shown in figures 7 and 8, were interpreted in terms of two types of water. The first, it was suggested, corresponds to a monolayer interacting directly with the protein. It appears to be characterized by a single dielectric relaxation time, τ_ϵ (of the same order as τ_r'), which is of the order of 10^{-9} s (298 K) and which has a negative enthalpy of activation. The second dispersion in ϵ', which only occurs at water contents above 0.3 g water/g protein was assigned to a second layer of water, less strongly influenced by the protein. The form of this dispersion suggested that the rotation of water molecules in this layer was characterized by a distribution of relaxation times, the average being close to 10^{-11} s at 298 K. Care was taken to discuss and eliminate all non-dipolar and non-aqueous contributions to ϵ' and ϵ'' and a model was proposed for the reorientation of water molecules in the first hydration layer

involving a transition state of increased local water structure, a sort of localized freezing. The dispersion corresponding to the first layer was not detectable below 250 K indicating freezing of the latter.

A study by Koenig & Schillinger (1969) of the ^{1}H spin-lattice relaxation dispersion (T_1 measured as a function of B_0) for aqueous solutions of the diamagnetic protein apotransferrin makes an interesting comparison with the dielectric study of the lysozyme/water system. Figure 9 shows a typical set of results.

It was found that all the measurements could be represented by the expression

$$R_1(\nu) = R_1(0)\left[\frac{0.463}{1+(2.51\nu/\nu_0)^2}+\frac{0.44}{1+(0.54\nu/\nu_0)^2}+\frac{0.093}{1+(0.025\nu/\nu_0)^2}\right]+R_{1w}, \qquad (17)$$

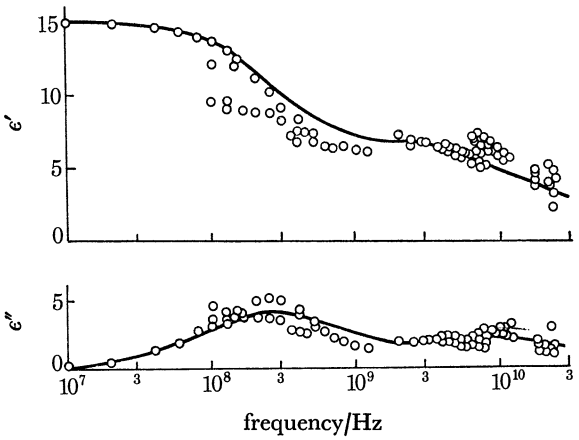

FIGURE 8. The variation with frequency of the dielectric constant (ϵ') and loss (ϵ'') for lysozyme containing 0.54 g water/g protein at 298 K. The full lines correspond to theoretically calculated values assuming two dispersions with relaxation times of 6.5×10^{-10} s and 1.6×10^{-11} s (Harvey & Hoekstra 1972).

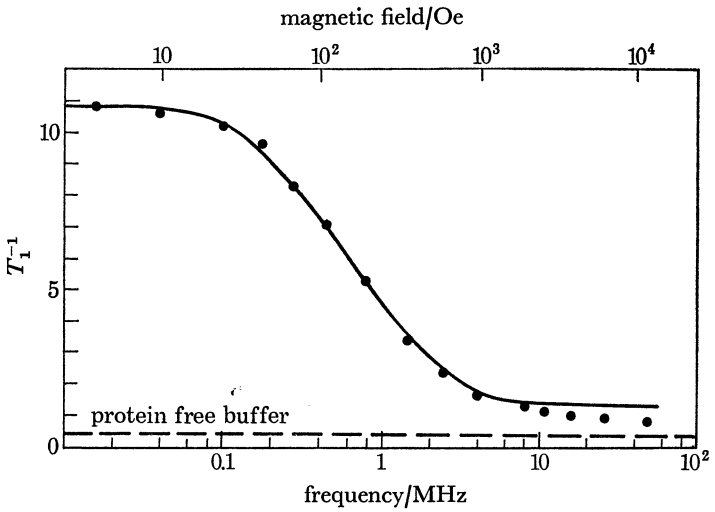

FIGURE 9. Water proton spin-lattice relaxation in an aqueous solution of the protein apotransferrin as a function of resonance frequency. The continuous line corresponds to equation (17) with the denominator of the third term taken as unity and clearly shows the existence of a dispersion at higher frequencies (Koenig & Schllinger 1969). 298 K, A_{280} = 171, 15 mass %, pH 7.7.

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where, at a given temperature, $R_1(\nu)$ and R_{1w} are the spin-lattice relaxation rates, $(T_1)^{-1}$, of the solution measured at frequency ν and of pure water, respectively. The characteristic frequency ν_0, dependent on the solution concentration, pH and temperature, was found to be in the range 0.23–1.28 MHz. This result was interpreted in terms of a small number of water molecules [between 10 and 70 per apotransferrin molecule, the number depending on whether the water molecules were assumed to be rigidly bound (all $\tau_r' \gg \tau_m$) or undergoing fast motion about only one axis relative to the protein surface (these were the only situations envisaged)] bound to and tumbling with the protein molecule, but exchanging with other, free, water molecules. The first two terms in equation (17) were associated with two correlation times, τ_m, for the anisotropic tumbling of the protein molecule while the third term was assigned to motion of a second class of more weakly bound water molecules. The τ_m values were deduced to be in the ranges 1.7×10^{-6}–3.1×10^{-7} s and 3.7×10^{-7}–6.7×10^{-8} s while the correlation time for the motion of the more weakly interacting molecules was calculated to be of the order of 1.7×10^{-8}–3×10^{-9} s, depending on the conditions.

An alternative interpretation of these results can be given in terms of the model discussed in §4*a* and *b*. This would envisage water molecules in the first hydration shell of the protein (a much larger number than 70) undergoing fast but anisotropic reorientations about all axes relative to the protein surface, the residual dipolar interaction being modulated by the slower, anisotropic tumbling of the protein. These bound water molecules then undergo exchange with free molecules at a rate fast compared to the T_1 of the bound molecules leading to a single T_1 for all the water protons but one which will show a weak dispersion when $\omega_0 \tau_r' \approx 1$ (see figure 2). The third term of equation (17) would be identified with this dispersion. Note that this gives $\tau_r' \approx 10^{-9}$ s which compares well with the dielectric relaxation time of the first hydration layer of lysozyme. In addition this interpretation has the merit of requiring only two types of water rather than three. Further detailed measurements would be needed to discriminate between these two possibilities.

(*b*) *Non-freezing water*

As was demonstrated by Kuntz (1969, 1971) cooling of many biological systems below the freezing point of ice leaves a fraction of the water which remains mobile. This is evidenced by its giving a relatively sharp ($T_2 \simeq 1$ ms) ^{1}H resonance spectrum (T_2 for ^{1}H in ice $< 10\,\mu$s). Several groups of workers have studied the proton relaxation behaviour of this non-freezing water in a number of systems such as striated muscle (Belton *et al.* 1973; Duff & Derbyshire 1974; Fung *et al.* 1975), hydrated collagen (Fung, Witschel & McAmis 1974), cornea (Bruynooghe & Packer 1975) and agarose gels (Duff & Derbyshire 1975). A typical set of results is shown in figure 10.

A T_1 minimum, dependent on ω_0, is observed and it is found that a single correlation time model is quite inadequate to represent the temperature and frequency dependence of T_1. In several cases (Duff & Derbyshire 1974, 1965; Belton *et al.* 1973; Fung *et al.* 1974, 1975) a log-normal distribution of correlation times (Resing 1965) has been found to represent the data although the calculated dipolar coupling strength (i.e. $\overline{\Omega_d^2}$ in equation (3)) is smaller than that arising from even the intramolecular proton–proton interaction in water and the values of T_2, calculated from the distribution parameters characterizing T_1, are an order of magnitude larger than those observed. There are a variety of possible explanations of these facts and it may be that each effect which has been discussed contributes but it seems likely (i) that there is a number or distribution of correlation times determining T_1 and these may arise because of the

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intrinsically anisotropic rotation of the bound water and/or because of the existence of binding sites with differing characteristics, (ii) that around 250 K the mean correlation time governing T_1 is of the order of 2×10^{-9} s and (iii) that because $T_{1\rho}$ ($\omega_1 \simeq 0.5 \times 10^6$ rad s^{-1}) is equal to T_2 for the protons in the non-freezing water in muscle (Duff & Derbyshire 1974) but is intermediate in value between T_1 and T_2 in the frozen agarose/water system (Duff & Derbyshire 1975), the extra contribution to T_2 arises from a process with a correlation time of the order of 10^{-6} s and is probably one or more of those described in figure 6 by the correlation times τ_e, τ_e' and τ_e''. Note that again the correlation time τ_r' for the reorientation of the bound water is of the order of 10^{-9} s.

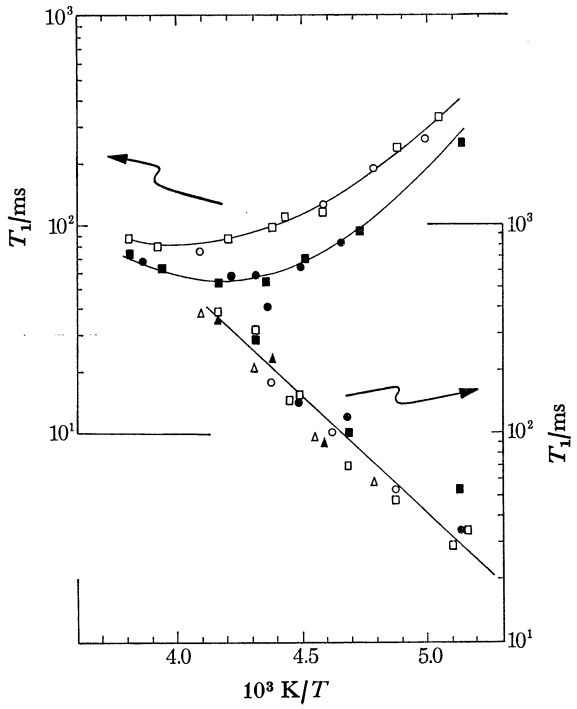

FIGURE 10. The variation with temperature of the spin-lattice and transverse relaxation times, T_1 and T_2, of the protons in the non-freezing water in frog gastrocnemius muscle. □, ○, △, different muscles, $(\omega_0/2\pi) = 60$ MHz; ■, ●, ▲, different muscles, $(\omega_0/2\pi) = 30$ MHz. (Belton *et al.* 1973.)

There is direct evidence for the existence of dynamically oriented water in striated muscle from ^{2}H n.m.r. measurements (Belton *et al.* 1975). As shown by Woessner & Snowden (1969*a*, *b*), intermolecular deuteron exchange, unlike the corresponding process for protons, is ineffective in averaging any splitting, ω_q, arising from dynamically oriented D_2O and both ω_q and ω_d splittings are completely refocused by a $90^\circ_{x'}$–τ–$90^\circ_{y'}$ pulse sequence. Figure 11 shows the variation with pulse separation τ of the ratio of the spin echo amplitudes obtained from $90^\circ_{x'}$–τ–$180^\circ_{y'}$ and $90^\circ_{x'}$–τ–$90^\circ_{y'}$ pulse sequences applied to ^{2}H nuclei in D_2O exchanged frog gastrocnemius muscle.

The implication of this result is that some fraction ($\sim 20\,\%$) of the D_2O molecules in this system are in slow exchange (on a time scale of T_2–tens of milliseconds) with the rest of the D_2O molecules and, on average, experience a non spherically symmetric environment. The residual ^{2}H quadrupole splitting, ω_q, for this fraction is only a few hertz which is very small compared to the values observed in highly ordered biological and related systems (see §5*c*).

However, we should note that, over the time scale available for this averaging process (~ 10 ms), assuming a reasonable value for D_s, the self-diffusion coefficient of the D_2O molecules in this system, a typical deuterium nucleus will have moved a distance comparable to the dimensions of the sarcomere. Bearing in mind the considerable distribution of angles θ (see figure 6) that the non-aqueous components of the muscle will present to the water molecules within the sarcomere, it is not surprising that the residual, diffusion averaged, anisotropy is small. The important point is that it is present and on a much shorter timescale could represent a significant contribution to the relaxation processes, particularly T_2 and $T_{1\rho}$.

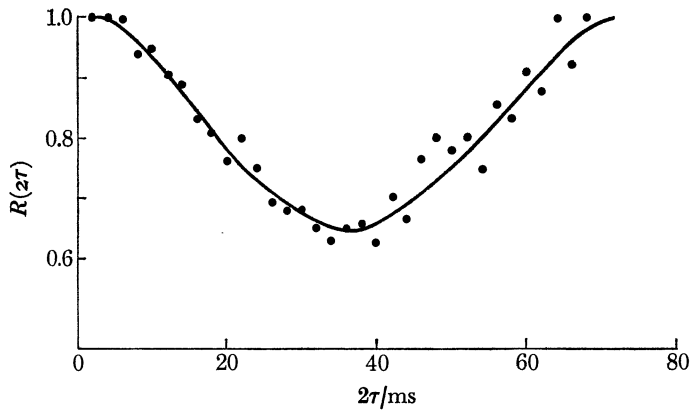

FIGURE 11. $R(2\tau)$ as a function of 2τ for ^2H nuclei in D_2O-exchanged frog gastrocnemius muscle at 293 K. $R(2\tau)$ is the ratio of the deuterium spin echo amplitude at time 2τ observed with a $90^\circ_{x'}-\tau-180^\circ_{y'}$ pulse sequence to twice the equivalent spin echo amplitude measured with a $90^\circ_{x'}-\tau-90^\circ_{y'}$ pulse sequence. The continuous line indicates the type of behaviour expected for a system in which the ^2H nuclei exist in three distinct relaxation environments only one of which experiences a residual quadrupolar splitting.

(*c*) *Dynamically oriented water*

We now look at some systems which clearly show the presence of dynamically oriented water, for example hydrated tendon collagen (Berendsen 1962; Berendsen & Migchelsen 1966; Dehl & Hoeve 1969; Chapman & McLauchlan 1969; Chapman, Campbell & McLauchlan 1970; Chapman, Danyluk & McLauchlan 1971; Fung & Trautmann 1971; Fung & Siegel 1972; Dehl 1973; Fung & Wei 1973; Migchelsen & Berendsen 1972), cornea (Bruynooghe & Packer 1975), phospholipid/water dispersions (Finer 1973; Gottlieb, Inglefield & Lange 1973), hydrated clays (Woessner & Snowden 1969*a*, *b*; Woessner, Snowden & Meyer 1969, 1970; Woessner & Snowden 1973; Woessner 1974; Woessner 1975) and soap–water lamellar mesophases (Johansson & Drakenberg 1971).

We start with tendon collagen which, for our purposes, we can consider to have a uniaxial, fibrillar structure. Neglecting a broad resonance arising from rigid parts of the collagen molecules, the proton n.m.r. spectrum of hydrated collagen consists of a doublet and a singlet while the ^2D spectrum of D_2O-hydrated collagen is only a doublet. The doublets (^1H or ^2H) arise from dynamically oriented water and the doublet splitting, $\omega_{d,q}$, varies as $(3\cos^2\theta - 1)$, where θ is the angle between the collagen fibre axis and the field $\boldsymbol{B}_0$ and increases with decreasing water content (Fung & Siegel 1972; Migchelsen & Berendsen 1972). The single line in the proton spectrum has an angle dependent width and is generally regarded as arising from mobile protons of the collagen molecules (Dehl & Hoeve 1969; Migchelsen & Berendsen 1972). The proton doublet can be made to collapse by raising the temperature, increasing the water

content or by adding certain salts (Berendsen & Migchelsen 1966; Migchelsen & Berendsen 1972). This is due to intermolecular proton exchange ($\tau_e \sim 10^{-4}$ s at 298 K). The widths of the proton doublet lines at lower temperatures have been shown to be dominated by the intramolecular dipolar interactions and were interpreted in terms of rotational motions although modulation by an exchange process was considered (Migchelsen & Berendsen 1972; Chapman *et al.* 1971). At water contents in the range 0.2–0.5 g water/g dry collagen the values of τ_r' deduced, assuming a single correlation time, are of the order 10^{-7} s at 298 K (Migchelsen & Berendsen 1972). Both the values of T_2 used to deduce this correlation time and the method used to calculate it have been criticized by Fung *et al.* (1974). These workers showed that for collagen containing < 0.5 g water/g collagen the water was not capable of being frozen and that T_1 minima were obtained for these non-freezing water protons at temperatures in the range 220–250 K for frequencies between 5 and 6 MHz. This again implies a median correlation time of the order of 10^{-9} s at 250 K which could relate directly to the reorientation of bound water molecules.

There is still considerable disagreement on the detailed model for water in collagen. Consideration of the available n.m.r. data and the measured anisotropy in the dielectric properties of collagen led Chapman *et al.* (1971) to support a model, involving chains of tetrahedrally hydrogen-bonded water molecules sited in the interchain grooves of the collagen triple helix, originally proposed by Berendsen (1962). More recently, Migchelsen & Berendsen (1972) have suggested that other models involving specifically bonded water molecules may be more consistent with the n.m.r measurements. Yet another detailed model has been proposed by Hoeve & Lue (1974) on the basis of dielectric relaxation measurements at low water contents. It is clear that further careful work on this system is necessary to establish the correct model but it would seem that the water in collagen shows several similarities with that in other biological materials.

A closely related system to tendon collagen is the corneal stroma. This consists of a stack of lamellae, each of the order of 2 μm in thickness and each consisting primarily of a uniaxial arrangement of hydrated collagen fibrils. The orientation of the fibre axis in the lamellar plane changes by large angles between adjacent lamellae. The water content *in vivo* is typically of the order of 4 g water/g dry stroma. At this water content the proton resonance is virtually a single line but reduction of the water content by equilibration at low relative humidities or by cooling below 260 K leads to a proton resonance typical of the collagen/water system as described above. Figure 12 shows the temperature dependence of the proton free induction decay signal (the Fourier transform of the normal spectrum (Farrar & Becker 1971)) for corneal stroma with a water content of *ca.* 0.8 g water/g dry stroma and with $\boldsymbol{B}_0$ perpendicular to the lamellar plane.

As for hydrated tendon collagen the dipolar doublet is collapsed at high temperature due to intermolecular proton exchange and is broadened beyond detection at low temperatures (Bruynooghe & Packer 1975). The non-freezing water in corneal stroma exhibits a proton T_1 minimum at 245 K for a resonance frequency of 60 MHz, again indicating a mean correlation time, τ_r', close to 10^{-9} s this temperature. The implications are that it is probable that the model outlined in figure 6 can encompass all these systems. This, however, remains to be established.

Water in clays is dynamically oriented and Woessner has made a detailed study of many aspects of the proton and deuteron n.m.r. properties of water in these systems, making particular

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use, as we have already noted, of two-pulse spin echo sequences (Woessner & Snowden 1969*a*, *b*, 1973; Woessner *et al.* 1969, 1973; Woessner 1974, 1975). Clays are layer silicates which can, in many cases, accommodate water molecules between the layers with thicknesses varying from a monolayer upwards. The ^{1}H and ^{2}H n.m.r. spectra of these systems show doublets with splittings which indicate dynamic orientation of, possibly, only the first layer or two of water with diffusional exchange leading to a linear dependence of the splittings on water content (Woessner & Snowden 1969*a*). ^{1}H T_1 measurements at 25 MHz for samples with water contents in the range 0.09–0.32 g water/g dry clay show minima in the temperature range 210–250 K.

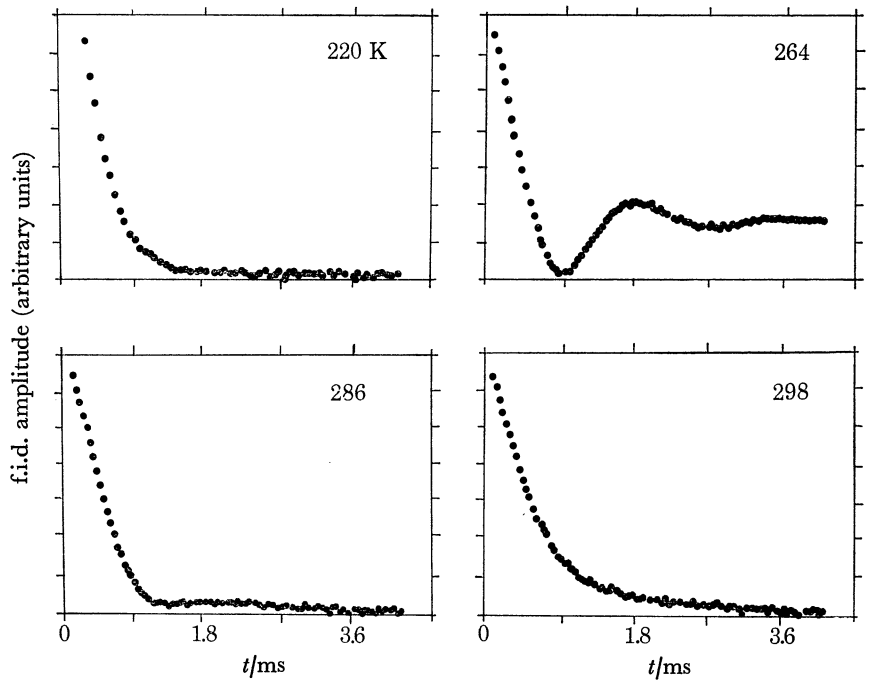

FIGURE 12. The temperature dependence of the ^{1}H free induction decay signal from bovine corneal stroma at a water content of *ca.* 0.8 g water/g dry tissue. At intermediate temperatures the signal corresponds to a spectrum consisting of a doublet and a singlet. The doublet arises from dynamically oriented water, the singlet from mobile collagen protons. At high temperatures the doublet collapses to a singlet because of proton exchange between water molecules while at low temperatures increased transverse relaxation broadens the lines to obscure the doublet splitting (Bruynooghe & Packer 1975).

These results again indicate the presence of at least one type of motion (τ_r'?), dominating T_1, which has an average correlation time of the order of 10^{-9} s. In this context, an interesting observation made by Woessner is that the ratio of the deuteron to proton splittings, for water oriented by clay surfaces, is close to 3.75 and that, under comparable conditions of water content, temperature etc., this ratio seems insensitive to the type of clay. He also points out that much the same ratio between these splittings exists for water oriented in collagen, lithium-DNA and rayon (Woessner 1975). This rather surprising fact suggests the possibility that it is merely the presence of a 'static' surface and not its nature that matters in producing dynamic orientation of the water and that water–water interactions are dominant in determining the details of that dynamic orientation. Finally we note that intermolecular proton exchange occurs in hydrated clays with lifetimes of the order 0.7–13 ms depending on clay type, cation etc. (Woessner 1974).

Before leaving clays we should mention studies of the proton self-diffusion in these systems y neutrons cattering (Stirling & White 1971; Olejnik & White 1972). It was found that 1/lg D_s aried linearly with the inverse of the water layer thickness and this was interpreted as ndicating that the dynamic ordering effect of the clay surface had a correlation length of about nm, measured from the surface (later reported as 0.5 nm (Hayter *et al.* 1974)). The high nobility of the interlayer water ($D_s = 5 \times 10^{-11}$ m^2 s^{-1} for a monolayer, indicating an r.m.s. isplacement of 500 nm in 1 ms) and the short correlation length for dynamic ordering is in ccord with the general picture of the water dynamics and ordering outlined in figure 6.

A similar neutron scattering study of the water in the lamellar phase of the ammonium erfluoro-octanoate/water system (Hayter *et al.* 1974) indicated that the dynamic ordering effect ecayed much faster with distance from the lamellar soap/water interface than in the clay ystem, a characteristic distance of only 0.2 nm being deduced. This is consistent with the bservations that, in soap/water systems at comparable water contents, the deuteron n.m.r. plittings (observation of which confirms the presence of dynamically oriented water) are onsiderably smaller than those in clay/water systems (Johansson & Drakenberg 1971) and hat the water relaxation times are much longer (Ahmad 1975; Packer & Ramsden 1975).

Lamellar soap/water or phospholipid/water mesophases form useful models for the types of ystem encountered in many biological materials. Studies of ^{2}H n.m.r. doublet splittings in ecithin/D_2O lamellae (Finer 1973) have demonstrated the existence of distinguishable hydra- ion sites and led to the conclusion that the observed splitting arose primarily from the inter- ction of one water molecule with the phospholipid phosphate head-groups, this molecule eing in fast exchange with other water molecules. A study of ^{1}H relaxation in the same system vas interpreted in terms of an exchange process ($\tau_e' \approx 6 \times 10^{-5}$ s at 293 K) and it was con- luded that some six water molecules were bound to each lecithin molecule (Gottlieb *et al.* 973). Again, the basic model of figure 6 seems to be applicable.

The value of τ_e' found by Gottlieb *et al.* (1973) for the lecithin/water system is similar in nagnitude to values obtained in other systems. Measurements of ^{1}H, T_1, $T_{1\rho}(\omega_1)$ and $T_2(t_p)$ n the lamellar phase of cesium perfluoro-octanoate clearly show dispersions in the region orresponding to correlation times of the order 10^{-4}–10^{-6} s (Packer & Ramsden 1975). It ppears that two correlation times are required to explain the form of this dispersion, one of the rder 10^{-5} s, the other 10^{-6} s, and it is suggested that the shorter of these corresponds to the ound-free exchange process characterized by τ_e' in figure 6.

(*d*) *Biological tissues*

The first report of the observation of this surface-bulk exchange process was made by Pintar nd his co-workers when studying proton relaxation in tissues. They initially reported mea- urements of a $T_{1\rho}$ dispersion for the water protons in striated muscle (Thompson, Knispel & intar 1973). In a later publication the same workers (Knispel, Thompson & Pintar 1974) eported measurements of T_2, $T_{1\rho}$ ($6 \times 10^3 < \omega_1 < 6 \times 10^5$ rad s^{-1}) and T_1 ($10^8 < \omega_0 < 2.9 \times 10^8$ rad s) for the protons in a number of tissues. A typical set of results is shown in figure 3.

In order to interpret these measurements it was found that a model involving a minimum of hree correlation times has to be used. These were, in the nomenclature of figure 6

(i) τ_e'–bound-free water exchange (originally attributed to intermolecular proton exchange Thompson *et al.* 1973) but later reassigned to this process (Knispel *et al.* 1974)).

(ii) τ_r'–rotation of bound water molecules and

(iii) a single correlation time describing the rotational/translational diffusional motions of the free water, i.e. a combined value covering τ_r and τ_D. The equations used to interpret the results were of the form

$$(T_1)^{-1} = p_b BK(\omega_0\tau_r') + (1-p_b)\,C, \tag{18}$$

$$(T_{1\rho})^{-1} = p_b A\,\frac{\tau_e'}{(1+4\omega_1^2\tau_e'^2)} + p_b BL(\omega_0\tau_r') + (1-p_b)\,C \tag{19}$$

and

$$(T_2)^{-1} = p_b A\tau_e' + p_b BL(\omega_0\tau_r') + (1-p_b)\,C, \tag{20}$$

where

$$K(\omega_0\tau_r') = \left[\frac{\tau_r'}{1+\omega_0^2\tau_r'^2} + \frac{4\tau_r'}{(1+4\omega_0^2\tau_r'^2)}\right]$$

and

$$L(\omega_0\tau_r') = \left[\tfrac{3}{2}\tau_r' + \frac{5}{2}\left\{\frac{\tau_r'}{(1+\omega_0^2\tau_r'^2)}\right\} + \left\{\frac{\tau_r'}{1+4\omega_0^2\tau_r'^2}\right\}\right].$$

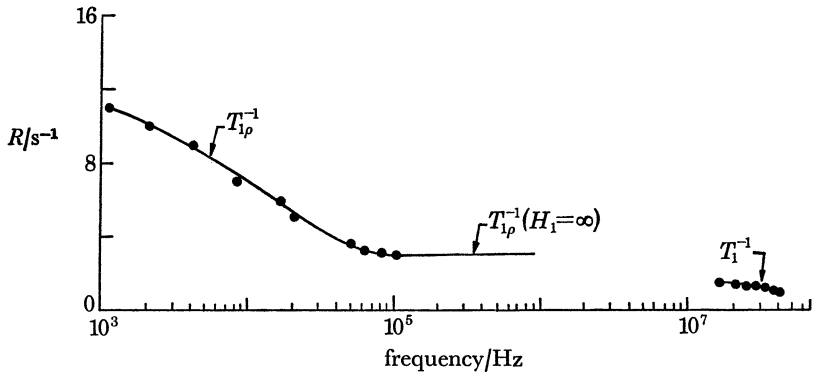

FIGURE 13. The variation with frequency of $T_1(\omega_0)$ and $T_{1\rho}(\omega_1)$ for water protons in a tissue sample (mouse tumour). The full line represents a fit of the data to a model involving three correlation times corresponding to τ_e', τ_r' and $\tau_{r/D}$ in figure 6 (Knispel & Pintar 1974).

p_b is the fraction of the water which is bound, C the relaxation rate of free water (for which it is assumed $T_1 = T_{1\rho} = T_2$) and A and B are parameters representing the effective strength of the nuclear interactions modulated by τ_e' and τ_r' respectively. Typical values obtained for these various parameters were (for mouse muscle) $p_b A$, 1.3×10^6 rad^2 s^{-2}; τ_e', 7×10^{-6} s; $p_b B$, 12×10^8 rad^2 s^{-2}; τ_r', 2.3×10^{-8} s and $(1-p_b)\,C$, 1.25 s^{-1}. Note that the exchange correlation time, τ_e', is of the order of 10^3 times τ_r' and that the latter is in the region of 10^{-9} s although a little longer. In fact, because T_1 for the water protons in muscle increases with increasing temperature at $\omega_0 = 12.5\times10^7$ rad s^{-1} (Finch & Homer 1974) we can deduce that, if as Knispel *et al.* (1974) suggests, T_1 at such a frequency is dominated by the rotation of the bound water, then τ_r' must be $< 5\times10^{-9}$ s^{-1}. A factor of four, however, is not too significant in this context since the models used are undeniably oversimplified. A minimum value of 0.01 was deduced for p_b which, if it were the actual value would imply that (i) the strength of the interaction modulated by rotation in the bound state (presumably dipolar coupling) is rather higher than seems reasonable ($B = 12\times10^{11}$ rad^2 s^{-2} compared with 5.4×10^9 rad^2 s^{-2} for the intramolecular proton dipolar coupling in water), (ii) the value of C is of the order of three times that for pure bulk water at the same temperature and (iii) of the order of 1% of the protons effectively 'cool' the rest in terms of spin-lattice relaxation. The basic conclusion that at least three correlation times are required is clearly established by this work.

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Another approach to the description of the dynamics of water in tissue like striated muscle is exemplified by a study by Finch & Homer (1974). They fitted 61 measurements of T_1, $T_{1\rho}$ and T_2 for the water protons measured at various values of ω_0, ω_1 and temperature to equations (3), (4) and (5) with the added assumption of a discrete distribution of correlation times. They found that their data were best fitted by a distribution involving five correlation times as illustrated in figure 14.

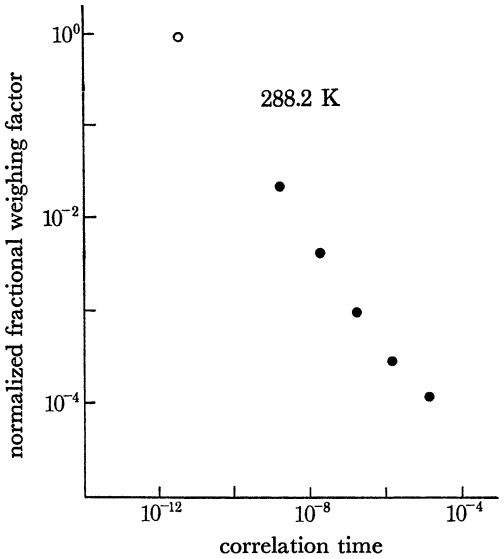

FIGURE 14. The discrete distribution of correlation times which best describes measurements of the water proton relaxation times (T_1, $T_{1\rho}$, and T_2) in frog gastrocnemius muscle according to Finch & Homer (1974). ○, free water; ●, bound or restricted water.

It can be seen that the general conclusion is the same as from the previous approach in that a small fraction (< 3 %) of the protons have reduced correlation times compared with bulk water. However, the main problem with this approach is that, as has been pointed out before (Belton *et al.* 1973), fitting to an arbitrary distribution of correlation times may just represent a convenient parameterization of the experimental data and that the criterion of a good fit to a particular model is no guarantee of the validity of that model. Perhaps the weakest assumption of this distribution of correlation times approach is that all motional processes modulate the same interaction strength. The evidence presented throughout this paper would seem to suggest that this is not so.

In the studies of tissues just discussed the nuclear magnetic relaxation behaviour was observed to be close to or indistinguishable from a single exponential process. Allowing for the well established heterogeneity of these materials it must be concluded that fast diffusional averaging occurs in these systems (see figure 5 and §4*b*). In many tissues, however, this is not so and more complex relaxation behaviour is observed. We now look briefly at the types of effect which can be seen and at the information these contain.

Belton *et al.* (Belton, Jackson & Packer 1972; Belton & Packer 1974) observed that the water proton transverse relaxation in the gastrocnemius muscle of the frog *Rana temporaria* was best represented by three exponential processes. The longest relaxation time component, amounting to 12–15 % of the total, was assigned as extracellular water. Similar behaviour has been observed by other groups (Hazelwood, Chang, Nichols & Woessner 1974; Civan & Shporer

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1975). It is difficult to prove that this assignment is correct but more recent work on the single-celled muscle from the giant barnacle (*Balanis nubilis*) and whole cornea tends to support it. The barnacle muscle, being single-celled, has no intercellular space as such but it does have deep clefts in its cell membrane which penetrate into the cell and these would be expected to contain water akin to water in the intercellular spaces of a multicelled muscle. Observations of the proton transverse relaxation of barnacle muscle cells equilibrated with iso-, hyper- and hypotonic Ringer solutions showed a long time component amounting to 4%, 0% and 8% of the total, respectively (Belton & Packer 1975). These percentages are close to those found for the cleft spaces in these muscles under these conditions from other measurements. The remaining parts of the transverse relaxation decays required at least two components for their description, the relative proportions of these being quite different to the corresponding components for frog gastrocnemius muscle. The origin of these other two components has not been clearly established and neither have the reasons for the observation of multi-exponential relaxation behaviour in some cases but not in others.

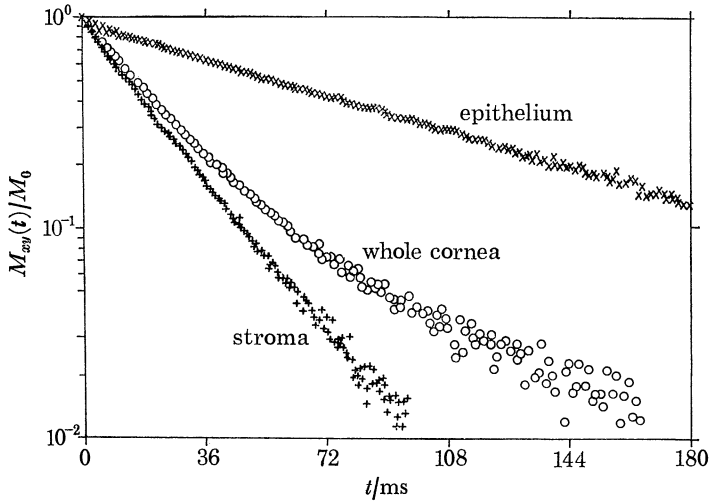

FIGURE 15. ^{1}H transverse relaxation in bovine cornea. ○, whole cornea; +, corneal stroma; ×, epithelial cells. It can be shown that the relaxation in the whole cornea is made up of a superposition of the two separate curves with appropriate weightings (Bruynooghe & Packer 1975).

A case in which it has proved possible to unambiguously identify two relaxation components as arising from two spatially distinguishable regions is that of cornea. In whole cornea the stroma (described above) is bounded on two sides by layers of epithelial and endothelial cells. It is possible to separate these from the stroma by dissection. Figure 15 shows the proton transverse relaxation behaviour for whole cornea, stroma and epithelial cells (Bruynooghe & Packer 1975). This demonstrates that the long time relaxation component observed in whole cornea arises from the water in the epithelial/endothelial cells and that it is possible to separate the total decay curve into a sum of two individual components characteristic of the spatially separate regions.

The existence of distinguishable nuclear spin relaxation components from intra- and extracellular water have opened up the possibility of studying the rate of water transport across cell membranes. Two approaches have been developed one based on the use of proton transverse

relaxation (Conlon & Outhred 1972) the other on ^{17}O spin-lattice relaxation (Shporer & Civan 1975). Both methods rely on the fact that interchange of nuclei between sites of different relaxation time causes changes in the measured relaxation times. In the first approach, manganese(II) ions are added to the extracellular fluid to lower its relaxation time and, provided these ions are substantially excluded from the intracellular water, the changes observed in the relaxation time of the intracellular water may be used to deduce the rate constant for proton exchange across the membrane. This method has been applied to water exchange across human erythrocyte membranes (Conlon & Outhred 1972) giving values for the exchange rate constant, k_e of the order of 120 s^{-1} at 310 K. This value was within 10 % of the value obtained by tracer techniques. On the other hand, using ^{17}O-enriched water, Shporer & Civan (1975) found that the spin lattice relaxation of the ^{17}O in human erythrocyte suspensions was two-component and that the longer relaxation time, extracellular component was shortened by exchange with the shorter relaxation time intracellular component. Two points of note are that (i) spin-lattice relaxation was able to be used because the very much shorter relaxation times of the quadrupolar ^{17}O nucleus, compared with protons under comparable conditions, produced slow-exchange conditions (see §4*b*) and (ii) that no addition of materials such as Mn^{2+} was required. Both these points are significant in that spin-lattice relaxation measured at normal n.m.r. frequencies in the MHz range is much less subject to additional effects than is transverse relaxation. Shporer and Civan point out that Mn^{2+} ions cause a chemical shift in the protons of water molecules and that exchange between the two regions, with and without Mn^{2+} ions, could contribute significantly to the proton transverse relaxation. Similarly, the passage of a proton through the membrane itself could lead to a contribution to T_2 but would be very unlikely to contribute to T_1. It is not surprising therefore that the values for k_e obtained by ^{17}O T_1 measurements were smaller, being 43 and 71 s^{-1} at 298 and 310 K respectively. The activation energy was deduced to be of the order of 33 kJ mol^{-1} which is considerably higher than that for self-diffusion in water in this temperature range ($\sim$19 kJ mol^{-1}).

Conclusion

A substantial body of experimental evidence indicates that, in general, water molecules are rather mobile in biological tissues and other heterogeneous systems even at low water contents. The effects of proteins and other non-aqueous components seems to be to provide sites where the water molecules tumble anisotropically, correlation times for this motion being of the order of 10^{-9} s around 250–260 K. There may well be a number of different interactions involved which would lead to a range of such correlation times. It has, however, been suggested, that water–water interactions may play a dominant rôle in the dynamic orientation process, the nature of the substrate playing a minor rôle. Lifetimes of water molecules in the dynamically oriented 'bound' state are possibly of the order of 10^{-5}–10^{-6} s at around 300 K and it seems that the effects of the non-aqueous components on the water extend to only a distance of the order of one or two water molecule diameters. Water in excess of these few layers appears to have properties very similar to normal bulk water.

An unambiguous quantitative model for any particular system has yet to be obtained and it is clear that what is needed is a careful study of a number of well characterized systems, particularly using the broad range of nuclear spin relaxation techniques but also by other methods as applicable and appropriate.

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I would like to acknowledge the contributions made to my interest in and understanding of this area by Dr P. S. Belton, Dr T. C. Sellwood, Mr R. F. Bruynooghe and Mrs J. M. Ramsden. I also wish to acknowledge the generous support of my own work in this area by the Science Research Council and to thank the following journals for permission to reproduce the figures as indicated: *Biochim. biophys. Acta*, figure 10; *Biophys. J.*, figure 14; *Chem. Phys. Letts.*, figure 4; *J. biol. Chem.*, figure 9; *J. Magn. Resonance*, figure 13; *J. phys. Chem.*, figures 7 and 8.

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Discussion

D. A. T. Dick. (*Department of Anatomy, University of Dundee*). Have you measured correlation times for transfer between the layers of water on the surface of proteins in living cells when the cells are subject to osmotic swelling or shrinkage and have you found or would you expect an effect due to the water movement?

K. J. Packer. No we have not measured such correlation times as a function of osmotic swelling or shrinking. Whether or not these would depend on the water content changed in this way will depend on (*a*) the dimensions between successive macromolecular interfaces and (*b*) whether the osmotic insult causes changes in the state of aggregation of the macromolecular components and hence the detailed local morphology.

D. R. Wilkie. (*Physiology Department, University College London*). Is it known whether the 'non-freezing' or 'bound' water in tissues tends to exclude small solutes such as urea? If such solutes equilibrate to a uniform concentration throughout *all* the water this would resolve an old controversy about whether any of the water in muscle was bound, or whether it was all free as maintained by A. V. Hill.

K. J. Packer. To the best of my knowledge it is not established as to whether the non-freezing water in tissues excludes various ions and/or small solutes. The observation of an enhanced or reduced concentration of these materials in the non-freezing water itself would only be a pointer since one could not rule out the possibility that the freezing process itself caused the effects.

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